REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

I. CLAIM STATUS AND AMENDMENTS

Claims 1-3 and 5-19 were pending in this application when last examined

Claims 1-3, 7-9, 17 and 18 were examined on the merits and stand rejected.

Claims 5, 6, 10-16 and 19 were withdrawn as non-elected subject matter.

New claim 20 has been added. Support can be found in the disclosure, for example, at page 36, lines 11-18.

No new matter has been added.

Claims 1-3 and 5-20 are pending upon entry of this amendment.

The Specification at page 30, line 25 has been amended to recite "base sequence" instead of "amino acid sequence." Support can be found in throughout the Specification and original claims. For instance, support can be found in the first sentence of the paragraph at page 30, line 25, which recites "hybridizable to the base sequence." No new matter has been added.

II. ANTICIPATION REJECTION

In item 7 on pages 3-4 of the Office Action, claims 2-3, 7 and 17-18 were rejected under 35 U.S.C. § 102(b) as anticipated by Bell (US 5,436,155).

In the response filed July 12, 2006, claim 1 was amended to a nucleotide sequence, which hybridizes under high stringent conditions to the nucleotide sequence of SEQ ID NO: 6, wherein the high stringent conditions comprise a sodium concentration at about 19 mM and a temperature at about 65°C. It was argued that the recited hybridization language excludes the isolated nucleotide sequence in Bell having 14.6% identity to the nucleotide sequence of SEQ ID NO: 6, because the Bell sequence, which has such a low percent identity, would not hybridize under high stringent conditions to the claimed sequence.

In reply, the Office asserted that the claim lacks a recitation of washing conditions, and as such, the claim has been interpreted to encompass an infinite number of polynucleotides which

may hybridize to the nucleotide sequence of SEQ ID NO:6, including the nucleic acid molecule of Bell (which is 14.6% identical to SEQ ID NO:6).

This position is respectfully traversed.

Support for the "high stringent conditions" can be found in the disclosure at page 30, line 27 to page 31, line 8. In particular, see page 30, lines 27-35 of the Specification, which clearly discloses that hybridization techniques are well known and conventional in the field of biotechnology. Moreover, it is well known in the art that hybridization under high stringent conditions clearly requires a washing step. Accordingly, although the claims do not recite a "washing" step, such step is clearly encompassed by the "high stringent conditions" language as defined in the Specification and as understood by those skilled in the art.

In this regard, kindly note the large number of US patents which have been granted with the claim terminology "hybridizable under stringent condition", but without the wording "washing". Please see, for example, the claims of US Patent Nos. 7,125,977, 6,630,341 and 6,476,193, the front page and claims of which are attached herewith for the Office's convenience. Also, kindly note that many more patents with the similar claim terminology can be found by searching the USPTO patent database. This means that a skilled person recognizes that the wording "hybridizable" or "hybridizes" encompasses the washing step after the treatment under the stringent condition.

Further, it is well established in the field that sequences that hybridize under high stringent conditions are limited to the relatively few sequences that form the requisite number of base pairs over the hybridized nucleotide sequences. Such hybridizable nucleotide sequences will be structurally similar to the base sequence. Also, it is well established that sequences with low percent identity will not hybridize under high stringent conditions. Thus, it is respectfully submitted that the term "hybridizes under high stringent conditions" refers to hybridization and washing under conditions that permit only binding of a nucleic acid molecule, such as an oligonucleotide or cDNA molecule probe, to highly homologous sequences. Therefore, such "hybridization under high stringent conditions" language excludes sequences with low percent identity, such as the isolated nucleotide sequence in Bell having 14.6% identity to the nucleotide sequence of SEQ ID NO: 6.

Therefore, the rejection of claims 2-3, 7 and 17-18 under 35 U.S.C. § 102(b) is untenable and should be withdrawn.

In addition, please note new dependent claim 20 has been added. This claim further requires the hybridizable sequence of claim 2 to have "about 95% homology to the nucleotide sequence of SEQ ID NO:6." It is respectfully submitted that this new claim is not anticipated by the isolated nucleotide sequence in Bell, which has 14.6% identity to the nucleotide sequence of SEQ ID NO: 6. The sequence with 14.% identity in Bell is not a sequence with 95% homology. For this additional reason, the 102(b) anticipation rejection over Bell does not apply to new claim 20.

III. UTILITY & ENABLEMENT REJECTIONS

In item 8 on pages 5-8 of the Office Action, claims 1-3, 7-9 and 17-18 were rejected under 35 U.S.C. § 101 on the basis that the Specification fails to set forth a credible, specific and substantial asserted or well established utility for the claimed polynucleotide.

Consequently, claims 1-3, 7-9 and 17-18 were rejected under 35 U.S.C. § 112, first paragraph, on the basis that the Specification lacks enablement for how to use the claimed invention for essentially the same reasons set forth for the above 101 utility rejection. See item 9 on pages 8-13 of the Office Action .

These rejections are respectfully traversed.

In the utility rejection, the Office contends that the instant Specification does not teach any physiological significance or functional characteristics of the claimed human OT7T175 polynucleotide (SEQ ID NO:6) or polypeptide (SEQ ID NO: 5). The Office also asserts that the Specification does not disclose any working examples substantiating a specific activity of the polynucleotide of the present invention.

As noted on page 6 of the Action, the Specification discloses various uses of the claimed invention. For example, the Specification discloses: (1) design for antisense DNA (page 31, lines 22-33 though page 35, lines 1-19; (2) to treat diseases (page 6, lines 20-32, page 32, lines 4-5, pages 69-75, page 90, line 33 to page 91, line 7, and original claims 11-12); (3) to diagnose diseases (page 6, lines 20-32, page 32, lines 4-5, pages 69-75, page 90, line 33 to page 91, line 7,

and original claims 11-12); (4) to search for ligands, agonists and antagonists (page 51-55, page 75, lines 21-34 to page 81); and (5) to make transgenic animals for harvesting tissue and cells, which animals, tissues or cells can also be used for testing pharmaceuticals for cancer (page 100, lines 32-34 through page 102).

The Office discounted these utilities on the basis the Specification does not disclose diseases associated with upregulated, downregulated, mutated, deleted, or translocated hOT7T175; nor does it disclose the type of tissues or cell types in which the polynucleotide is abnormally expressed.

It is respectfully submitted that this position is unreasonable and inaccurate.

To satisfy the utility requirement under 35 U.S.C. § 101, the claims and the Specification must disclose either a "specific utility" or a "well-established utility" for the claimed invention, and such utility must be credible and substantial. M.P.E.P. § 2107.02.

A "specific utility" is an explicit statement/assertion of "why the applicant believes that the invention is useful" and is specific to the subject matter claimed. M.P.E.P. § 2107.02, A. Such statements will usually explain the purpose of how the invention may be used. Ibid.

An invention has a "well established utility" if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process) in view of the knowledge in the art, and (ii) the utility is specific, substantial and credible. M.P.E.P. § 2107.02, II, B; *Guidelines for Examination of Applications for Compliance With the Utility Requirement*, 66 Fed. Reg. 1097, 1098 (Jan. 5, 2001). In other words, it must be well known, immediately apparent and implied by the specification based on the disclosure of the properties of the claimed invention, either alone or taken with knowledge of one skilled in the art. A well-established utility is applicable to a broad class of the invention.

In the instant case, the Specification discloses a credible and substantial "specific" utility and a credible and substantial"well-established" utility for the claimed polynucleotide sequence of SEQ ID NO: 6, which encodes the protein of SEQ ID NO: 5.

The discovery process of the present invention is well summarized in the present Specification at page 5, line 27 to page 6, line 35. As described therein, the OT7T175 proteins

encoded by the polynucleotides of the present invention were identified as seven-transmembrane GPCR by hydrophobic plotting analysis. Then, the Applicants substantiated that the C-terminal peptide (metastin) of a protein encoded by the cancer metastasis-suppressor gene KiSS-1 possesses an activity of activating the rOT7T175 receptor protein. For instance, in Example 3 (1-2), the intracellular Ca ion concentration-increasing activity of the peptides (metastin) against rOT7T175 was measured using FLIPR. See the Specification at page 115, line 14 to page 118, line 23. The results revealed that metastin induced an increase in the intracellular calcium ion concentration specifically to the rOT7T175 expressing cells. The biological data are shown in FIG. 9.

The Specification further discloses that the peptides of the KiSS-1 gene products "are expected to suppress tumor metastasis." See the disclosure at page 6, lines 20-32 and original claims 11-12, which relate to methods of diagnosing and treating cancer.

From these previously known facts and the experimental results described in the present Specification, a skilled person would readily appreciate that the rat and human OT7T175 are associated with metastasis of cancer in view of the expected function of the newly-identified ligand, metastin, since it is the C-terminal peptide of a protein encoded by cancer matastasis-suppressor gene KiSS-1.

Thus, contrary to the Office's position, the Specification discloses a disease (i.e., cancer) associated with upregulated, downregulated, mutated, deleted, or translocated hOT7T175, and it does it disclose the type of tissues or cell types in which the polynucleotide is abnormally expressed.

In further support of this position, please see the Declaration, submitted during the prosecution of parent application, Serial No. 09/830,428, now US 6,669,965. This Declaration demonstrated intracellular Ca ion concentration-increasing activity of metastin against hOT7T175. A copy of the front page and the allowed claims of the parent application are enclosed herewith.

It is respectfully submitted that cancer diagnosis and cancer treatment are credible and substantial real world uses. Accordingly, it is respectfully submitted that the Specification describes at least a credible and substantial specific asserted and well established utility.

In this regard, the present invention has the following well established utilities **A & B** as evidenced by the attached scientific papers (References A-E). Although the attached references were published after the application date, they further substantiate the asserted and well established utilities of the present invention.

A. Relating to Prevention of Cancer Metastasis/Placenta related Function

The Ohtaki et al. reference (Nature, vol. 411., pp. 613-7, May 31, 2001 (Reference A)) is by the current Applicants. This reference is the original literature based on the present invention. The metastatis prevention activity of the present invention was substantiated in a metastasis model animal. In particular, this reference confirms that the carboxy-terminal 54 amino-acid residue peptide (known as known as metastin, which is the peptide of the instant invention) encoded by the KiSS-1 tumor suppressor gene inhibits chemotaxis and invasion of cancerous cells and it attenuates pulmonary metastasis of cancer cells. See, for instance, the first paragraph for this reference in column 2 on page 613.

The Masui et al. reference (Biochem Biophys Res Commun., vol. 315, pp. 85-92, Feb. 27, 2004, (Reference B)) is titled "Metastin and its variant forms suppress migration of pancreatic cancer cells" This paper further substantiated the metastasis prevention activity in a pancreatic cell line.

The Bilban et al. reference (J Cell Sci., Vol. 117(Pt 8), pp. 1319-28, Mar. 15, 2004 (Reference C)) is titled "Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts." This paper suggests that metastin (=kisspepptin) is associated with regulation of trophoblast in placenta.

B. Gonad Regulation related function

The Seminara et al. reference (Endocrinology, Vol. 147(5), pp. 2122-6, May 2006 (Reference D)) is titled "Continuous Human Metastin 45-54 Infusion Desensitizes G Protein-Coupled Receptor 54-Induced Gonadotropin-Releasing Hormone Release Monitored Indirectly in the Juvenile Male Rhesus Monkey (*Macaca mulatta*): A Finding with Therapeutic Implications." See for instance, the last sentence of the Abstract on page 2122, which states:

> The desensitization of GPR54 by continuous hu metastin 45-54 administration has therapeutic implications for a variety of conditions currently being treated by

GnRH and its analogs, including restoration of fertility in patients with abnormal GnRH secretion (*i.e.*, idiopathic hypogonadotropic hypogonadism and hypothalamic amenorrhea) and selective, reversible suppression of the pituitary-gonadal axis to achieve suppression of gonadal steroids (*i.e.* precocious puberty, endometriosis, uterine fibroids, and prostate cancer).

This paper further substantiates a functional activity of the present invention.

WO 2004/080479 (only first page with English abstract attached) (Reference E) describes that metastin relates to the regulation of gonadal function. It further states that metastin and its receptor are useful as gonadal function improving agents, promoters and inhibitors thereof, sexual secretion promoters and inhibitors, anti-infertility agents, anti-hormone sensitive cancer agents, and for screening.

From the foregoing, it is respectfully submitted that the specific asserted and well established utilities in the disclosure have been recognized by these in the art field as credible and substantial. In other words, these references further substantiate that the recited uses in the disclosure are credible and substantial.

Nonetheless, the Office asserts that specific or substantial utility cannot be recognized in treatment of diseases, diagnosis of diseases, etc. The Office also states that the hOT7T175 receptor polypeptide described therein is what is termed an "orphan protein" in the art (see page 7, last line to page 8, line 1 of the Office Action).

In reply, the orphan receptor is generally recognized as a receptor whose ligand has not been identified and thus no function is identified. However, the ligand of hOT7T175 was identified by the inventors in the present invention, and substantial utility was also identified by the inventors as described in the present Specification and the attached references.

Accordingly, it is respectfully submitted that the Office's position is unreasonable. Again, the Specification clearly discloses credible and substantial uses for the invention. Further, once the target polynucleotide/protein/polypeptide is identified for specific medical use (i.e., prevention of cancer metastasis in this case), a skilled person can readily use the target polynucleotide/protein/polypeptide for preparation of a pharmaceutical agent, diagnosis agent, etc. without undue burden.

A "substantial" utility defines a "real world" use. Practical utility is a shorthand way of attributing "real-world" value to claimed subject matter. M.P.E.P. § 2107, I. As a general matter, a reasonable correlation between the evidence presented and the asserted utility is sufficient to establish a credible and substantial utility. M.P.E.P. § 2107.03, I. See *Guidelines for Examination of Applications for Compliance With the Utility Requirement*, 66 Fed. Reg. 1097, 1098 (Jan. 5, 2001) and *Revised Utility Guidelines Training Materials*, published by the PTO for examination guidance, page 6.

It is respectfully submitted that cancer diagnostics and cancer therapeutics are "real world" uses. In other words, the specific asserted utilities and the well established utilities of the present application are substantial.

Furthermore, an assertion of utility is "credible" if it is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided. In other words, it is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion. M.P.E.P. § 2107.02, III, B; See also, *Guidelines for Examination of Applications for Compliance With the Utility Requirement*, 66 Fed. Reg. 1097, 1098 (Jan. 5, 2001). The credibility of a utility cannot be summarily dismissed by the Office.

In view of the totality of the evidence based on the disclosure and the state of art, those skilled in art would reasonably believe that the disclosed uses of the present invention for cancer diagnostics and cancer therapeutics are credible and substantial.

Lastly, it is well established that asserted utilities are ***presumed true***. See M.P.E.P. § 2107.01 and *In re* Brana, 34 U.S.P.Q.2d 1436, 1441 (Fed. Cir. 1995). To overcome the presumptive truth of the utilities set forth in the Specification, the Office must show by a ***preponderance of evidence*** that it is ***more likely than not*** that the asserted specific utility would be considered false by a person of ordinary skill. M.P.E.P. § 2107.01 and *In re* Corkill, 226 U.S.P.Q. 1005, 1008 (Fed. Cir. 1985).

It is respectfully submitted that the Office has not met this burden by providing arguments or evidence showing by a ***preponderance of evidence*** that it is ***more likely than not*** that the asserted specific utility would be considered false by a person of ordinary skill.

Thus, the Specification discloses both a credible and substantial "specific utility" and "well-established utility" for the claimed polynucleotide sequence of SEQ ID NO: 6 and the protein of SEQ ID NO: 5.

Furthermore, one of skill in the art, upon reading the disclosure and in view of the knowledge in the art, could make and use the present invention using conventional techniques without undue experimentation. For instance, diagnostic methods and techniques are conventional and routine in the art. Since the instant invention is useful as a cancer diagnostic, one of skill in the art could use the present invention to assess the level cancer metastasis without undue experimentation using conventional and routine techniques.

Therefore, the utility rejection under 35 U.S.C. § 101 and the enablement rejection of claims 1-3, 7-9 and 17-18 under 35 U.S.C. § 112, first paragraph, are untenable and should be withdrawn.

In item 10(i) on page 9 of the Office Action, claims 2-3, 7-9 and 17-18 were again rejected under 35 U.S.C. § 112, first paragraph, on the basis that the claims lack a recitation of a washing under stringent conditions. The Office contends that the claims recite an infinite number of polynucleotides that may hybridize for which the Specification is not enabling.

This rejection is respectfully traversed for the reasons discussed above.

Again, contrary to the Office's position, the language "hybridization under stringent conditions" includes a washing step, and thus, the claims does not encompass an infinite number of sequences. In fact, such the claim language encompasses relatively few sequences.

Therefore, the rejection of claims 2-3, 7-9 and 17-18 under 35 U.S.C. § 112, first paragraph, is untenable and should be withdrawn.

In item 10(ii) on pages 9-11 of the Office Action, it was indicated that claims 8-9 remain rejected under 35 U.S.C. § 112, first paragraph, on the basis that the Specification is not enabling for a diagnostic or therapeutic for all possible diseases except cancer.

This rejection is respectfully traversed for the reasons discussed above.

Thus, the Specification discloses both a credible and substantial "specific utility" and "well-established utility" for the claimed polynucleotide sequence of SEQ ID NO: 6 and the protein of SEQ ID NO: 5.

Furthermore, one of skill in the art, upon reading the disclosure and in view of the knowledge in the art, could make and use the present invention using conventional techniques without undue experimentation. For instance, diagnostic methods and techniques are conventional and routine in the art. Since the instant invention is useful as a cancer diagnostic, one of skill in the art could use the present invention to assess the level cancer metastasis without undue experimentation using conventional and routine techniques.

Therefore, the utility rejection under 35 U.S.C. § 101 and the enablement rejection of claims 1-3, 7-9 and 17-18 under 35 U.S.C. § 112, first paragraph, are untenable and should be withdrawn.

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This rejection is respectfully traversed for the reasons discussed above.

Again, contrary to the Office's position, the language "hybridization under stringent conditions" includes a washing step, and thus, the claims does not encompass an infinite number of sequences. In fact, such the claim language encompasses relatively few sequences.

Therefore, the rejection of claims 2-3, 7-9 and 17-18 under 35 U.S.C. § 112, first paragraph, is untenable and should be withdrawn.

In item 10(ii) on pages 9-11 of the Office Action, it was indicated that claims 8-9 remain rejected under 35 U.S.C. § 112, first paragraph, on the basis that the Specification is not enabling for a diagnostic or therapeutic for all possible diseases except cancer.

This rejection is respectfully traversed for the reasons discussed above. The claims are limited to cancer. For the reasons noted above, Applicants have demonstrated a sufficient nexus

between cancer and the present invention. Also, for the reasons noted above, it would not take undue experimentation to use the present invention in the routine procedures disclosed in the art to diagnose cancer.

Therefore, the rejection of claims 8-9 under 35 U.S.C. § 112, first paragraph, is untenable and should be withdrawn.

IV. WRITTEN DESCRIPTION REJECTION

In item 11 on pages 13-15 of the Office Action, claims 2-3, 7-9 and 17-18 were again rejected under 35 U.S.C. § 112, first paragraph, on the basis that the Specification lacks written description support for the genus of claimed polynucleotides due to the lack a recitation of a washing under stringent conditions.

This rejection is respectfully traversed for the reasons discussed above.

Again, contrary to the Office's position, the claim language "hybridization under stringent conditions" does not encompass a large number of sequences. Instead, such language encompasses relatively few sequences given that only highly homologous sequences will hybridize to the base sequence under high stringent conditions. Thus, it is respectfully submitted that one of skill in the art would believe that Applicants were in possession of a representative number of hybridizable sequences at the time of the invention.

Therefore, the rejection of claims 2-3, 7-9 and 17-18 under 35 U.S.C. § 112, first paragraph, is untenable and should be withdrawn.

CONCLUSION

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance and early notice to that effect is hereby requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

Respectfully submitted,

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ATTACHMENTS

A. Nature, Vol. 411, No. 6837, pp. 613-617, 2001;

B. Biochem Biophys Res Commun, Vol. 315, No. 1, pp. 85-92, 2004;

C. J Cell Sci, Vol. 117, Pt. 8, pp. 1319-1328, 2004;

D. Endocrinology, Vol. 147, No. 5, pp. 2122-2126, 2006;

E. WO 2004/080479 (first page and English abstract only);

F. Front page and claims for US Patent Nos. 7,125,977, 6,630,341 and 6,476,193; and

G. Front page and claims of parent application, Serial No. 09/830,428, now US 6,669,965;

Reference A (1)

homozygous F_2 or F_3 *mor1-1* segregants was used to map the *MOR1* locus using CAPS (cleaved amplified polymorphic sequence) markers[28] to distinguish parental (Columbia) from Ler DNA sequence. Tight linkage was established to two CAPS markers flanking (m323 and C43/C44), and to one CAPS marker (Ubi) located within, bacterial artificial chromosome (BAC) T20F21 (Arabidopsis Biological Resource Centre, GenBank accession no. AC006068) at ~67.19 cM on chromosome 2. Homology searches of predicted genes on BAC T20F21 identified the most likely candidate gene as AC006068.3. Genomic and RT-PCR product sequence analysis identified point mutations in both mutant alleles. Transformation of *mor1-1* homozygotes with a subclone of BAC T20F21 containing *MOR1* restored the wild-type phenotype at the restrictive temperature, confirming the identity of the candidate gene. The wild type sequence from RT-PCR analysis is deposited in GenBank (accession no. AF367246).

Sequence analysis

Manual adjustment of the ClustalW multiple alignment used the BioEdit[29] program with reference to previously published pairwise alignments[8–10,12].

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Correspondence and requests for materials should be addressed to G.O.W. (e-mail: geoffw@rsbs.anu.edu.au). Sequences are deposited in GenBank under accession no. AF367246.

Metastasis suppressor gene *KiSS-1* encodes peptide ligand of a G-protein-coupled receptor

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Metastasis is a major cause of death in cancer patients and involves a multistep process including detachment of cancer cells from a primary cancer, invasion of surrounding tissue, spread through circulation, re-invasion and proliferation in distant organs. *KiSS-1* is a human metastasis suppressor gene[1], that suppresses metastases of human melanomas[2] and breast carcinomas[3] without affecting tumorigenicity. However, its gene product and functional mechanisms have not been elucidated. Here we show that *KiSS-1* (refs 1, 4) encodes a carboxy-terminally amidated peptide with 54 amino-acid residues, which we have isolated from human placenta as the endogenous ligand of an orphan G-protein-coupled receptor (hOT7T175) and have named 'metastin'. Metastin inhibits chemotaxis and invasion of hOT7T175-transfected CHO cells *in vitro* and attenuates pulmonary metastasis of hOT7T175-transfected B16-BL6 melanomas *in vivo*. The results suggest possible mechanisms of action for *KiSS-1* and a potential new therapeutic approach.

During a search for novel G-protein-coupled receptors (GPCRs) using degenerate polymerase chain reaction (PCR) strategy we found a rat orphan receptor (rOT7T175) that was nearly identical to GPR54 (ref. 5). To identify the endogenous ligand of OT7T175, we established a stable CHO cell line (CHO/h175) expressing the human counterpart hOT7T175. Although hOT7T175 had 39.2% amino-acid identity to human galanin receptor GALR2 (ref. 6), the cells did not show any response to a panel of known peptides, including galanin and galanin-like peptide[7]. On the other hand, human placental extract induced a robust increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$) in CHO/h175 cells. After several steps of high-performance liquid chromatography purification for this activity, we isolated about 6 μg of pure peptide from 740 g of chorionic tissue (Fig. 1a, b).

The amino-terminal sequence obtained for the isolated peptide, GTSLSPPPESSGSRQQPGLXA (X not identified), was identical to the partial amino-acid sequence (Gly 68 to Ala 88) of the *KiSS-1* gene product[1,4]. Although the *KiSS-1* gene product has been proposed to be an intracellular signalling molecule such as an SH3 ligand or a substrate for protein kinase[1,4], it has an obvious secretory signal sequence[8] and two potential processing sites[9] (Fig. 1d). Processing at these sites followed by amide transfer[9] produces the *KiSS-1* gene-product (68–121) amide. Mass spectrometric analysis revealed that the *m/z* value of the isolated peptide (5,857.2) was very close to the theoretical value of the *KiSS-1* gene-product (68–121) amide (5,858.5). The *m/z* values of V8 protease-digested peptides (4,006.4 and 1,869.8) were in accordance with the theoretical value of the *KiSS-1* gene-product (68–106) amide (4,006.4) and that of the (107–121) amide (1,869.9), respectively. The isolated peptide was thus identified as the *KiSS-1* gene-product (68–121) amide and designated metastin.

Metastin and its derivative peptides were synthesized chemically or by a recombinant DNA method[10] (Fig. 1c). Metastin potently and specifically induced an increase in $[Ca^{2+}]_i$ in CHO/h175 cells (Fig. 2a). The C-terminally free (un-amidated) form of metastin showed only very weak activity. N-terminally truncated peptides, metastin (40–54) and (45–54) were three to ten times more active than metastin, whereas metastin (46–54) was less active. Thus, the C-terminally amidated sequence from Tyr 45 to Phe 54 is mostly involved in receptor interaction. Saturation binding analysis showed that [^{125}I-Tyr^{45}]metastin (40–54) binds to the membranes of CHO/h175 cells in a saturable manner with a K_d (dissociation constant) of 95 pM. The receptor binding affinity of each peptide was obtained by competitive binding analysis (Fig. 2b). The affinity of metastin (inhibition constant (K_i) = 0.34 nM) was high enough to show it to be a cognate ligand for hOT7T175. Although truncated peptides had higher receptor binding affinity than metastin, these peptides were not endogenous. The N-terminal portion of metastin is not essential for receptor binding, but it may be involved in another biological process such as stabilization and protection from proteolytic digestion.

We further studied whether metastin can suppress tumour metastasis. First, we tested the effect of peptide on cell motility, a feature that is considered to be crucial for invasion of cancer cells. The addition of metastin to the upper microchemotaxis chamber inhibited the chemotaxis of CHO/h175 cells towards fetal calf serum (FCS), but did not affect that of the CHO mock transfectants (CHO/mock) (Fig. 3a). The C-terminally free form of metastin had 10,000-fold less activity, which was in good agreement with the

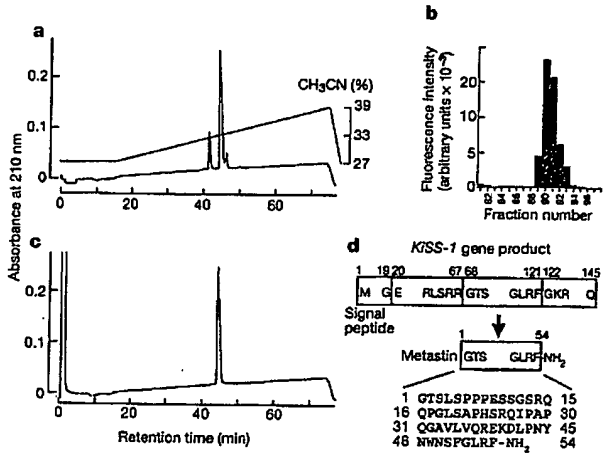

Figure 1 Purification of metastin. **a**, The elution profile of placental metastin at the final Super-ODS step. **b**, Each 0.5-min fraction of the eluate (from a) was collected and analysed by FLIPR assay with CHO/h175 cells. **c**, The elution profile of recombinant metastin under the same conditions as a. **d**, Hypothetical processing pathway of metastin in neuroendocrine tissues. After removal of the signal peptide Met 1 to Gly 19 (predicted by the SignalP server[8]), metastin precursor was cleaved at Arg 67 and at Arg 124 by a prohormone convertase. The C-terminal extended Arg 124 and Lys 123 were sequentially removed by carboxypeptidase, and Phe 121 was transamidated with Gly 122.

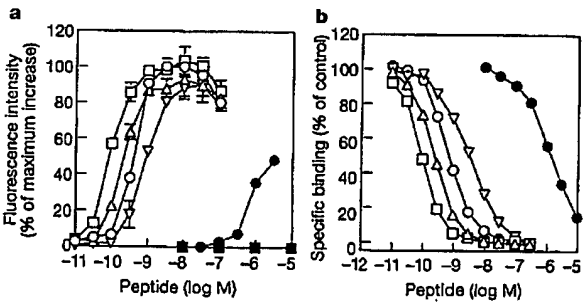

Figure 2 Pharmacological characterization. **a**, Response of CHO/h175 cells to metastin (open circle), metastin (40–54) (open uptriangle), (45–54) (open square), (46–54) (open down triangle) and metastin free form (filled circle) was measured by FLIPR assay. Response of CHO/mock cells to metastin (filled square). **b**, Competitive binding analysis using 100 pM [^{125}I-Tyr^{45}] metastin (40–54). Symbols are the same as in a. The K_i values[17] were: metastin, 0.34 nM; metastin (40–54), 0.10 nM; (45–54), 0.042 nM; (46–54), 1.5 nM; and free form, 640 nM.

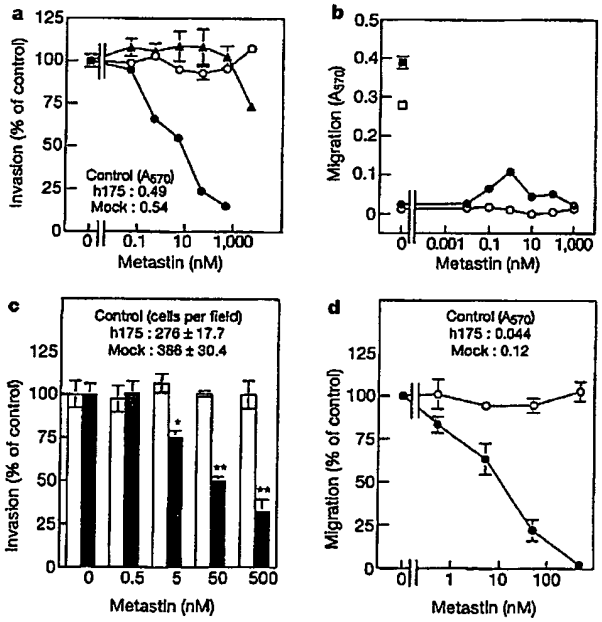

Figure 3 Cell migration and invasion. **a**, Migration of CHO/h175 (filled symbols) and CHO/mock cells (open symbols) towards FCS in the presence of metastin (circles) or its free form (triangles). **b**, Migration of CHO/h175 (filled symbols) and CHO/mock cells (open symbols) towards metastin (circles) or FCS (squares) for 4 h. **c**, Invasion of CHO/h175 (filled bars) and CHO/mock cells (open bars). **d**, Migration of B16-BL6/h175 (filled circles) and B16-BL6/mock melanomas (open circles) towards fibronectin. Values are mean ± s.e. (**a**, **b**, **d**) or mean ± s.d. (**c**). *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test).

pharmacological profile. Metastin by itself exerted weak chemotactic activity on CHO/h175 cells, but the maximum extent was only one-fourth of that induced by FCS and the activity disappeared at higher concentrations (10–1,000 nM) (Fig. 3b). In an *in vitro* invasion assay that tests migration through a Matrigel-coated porous filter, metastin inhibited FCS-induced invasion of CHO/h175 cells specifically with an IC_{50} value of about 50 nM (Fig. 3c).

We transfected hOT7T175 to B16-BL6 mouse melanoma and obtained a stable high expression clone (B16-BL6/h175) that showed $[Ca^{2+}]_i$ response to metastin. Metastin inhibited the fibronectin-induced chemotaxis of B16-BL6/h175 melanomas but not of the mock-transfected melanomas (B16-BL6/mock) (Fig. 3d). Next, we found that metastin induced excessive formation of focal adhesions (vinculin-positive patches) and stress fibres in B16-BL6/h175 melanomas (Fig. 4a, b). The cells started to express focal adhesions 5 min after the addition of metastin, and most of the cells displayed focal adhesions densely after 30 min. Excessive stress fibres were formed after 30 min but not after 5 min. Furthermore, metastin induced the phosphorylation of focal adhesion kinase (FAK) and paxillin (Fig. 4c, d), which is believed to be essential for the formation of focal adhesions[11]. The phosphorylation of FAK occurred rapidly and almost ceased after 10 min, whereas that of paxillin lasted longer. We hypothesize that metastin attenuates cellular motility by excessively inducing the adhesive phenotypes. The detailed signal transduction mechanisms should be studied in future. Sphingosine-1-phosphate is also known to inhibit the migration of B16 melanoma[12], which is mediated by a GPCR named Edg-5 and involves subsequent activation of Rho, FAK and paxillin, and overexpression of focal adhesions[13].

In spontaneous pulmonary metastasis assays, B16-BL6/h175 melanomas were injected into the footpads of mice on day 0 (d0), tumours were surgically excised on d21, and metastasized tumour foci were counted macroscopically on d35. As the lung had only a small number of metastasized cells (microscopic inspection) on d21, administration of metastin with an osmotic mini-pump, limited to two weeks, was started on d18. This was also to mimic a potential clinical setting. When tumours were surgically removed on d21, there were not significant differences in tumour size of metastin- and vehicle-treated mice (Table 1). On d35, metastin-treated mice had a marked decrease in the number of metastasized tumour foci compared with mice that had received vehicle (Table 1). The number of microscopic metastases also decreased in the metastin-treated mice (see Supplementary Information). On the other hand, metastin did not affect metastasis of B16-BL6/mock melanomas (Table 1). Therefore, the observed effect on tumour metastasis is probably due to the direct action of metastin on tumour cells, although metastin may have also exerted some pharmacological effect on host mice. On the basis of the inhibitory activity on cell migration and invasion *in vitro*, metastin can be considered to have caused the inhibitory activity on the invasion of primary tumours and re-invasion of circulating tumour cells. Furthermore, metastin may have attenuated the microgrowth of metastasizing cells, as metastin was effective despite being administered relatively late (d18). By contrast, metastin failed to block the experimental metastasis (Table 1) and the growth of tumour mass *in vivo* (see Supplementary Information). However, these findings do not eliminate the possibility of metastin action at lower cell density. The precise mechanism of the antimetastatic activity remains to be elucidated.

Table 1 Metastasis of B16-BL6/h175 melanomas

Cell injection	Administration	Tumour size (mm^3)*	Tumour foci (no.)	*n*
B16-BL6/h175 (s.c.)	Metastin	900.8 ± 97.6†	13.0 ± 3.2‡	5
	Vehicle	1,037.2 ± 170.5	37.8 ± 2.5	4
B13-BL6/mock (s.c.)	Metastin	1,062.9 ± 81.7†	31.2 ± 6.6†	10
	Vehicle	1,135.2 ± 99.6	26.4 ± 6.8	8
B16-BL6/h175 (i.v.)	Metastin		59.8 ± 9.8†	6
	Vehicle		42.7 ± 6.9	6

*Tumour size on day 21.
† Not significant compared with vehicle.
‡ $P < 0.01$ compared with vehicle (Student's *t*-test). Values are mean ± s.e.

We investigated the tissue distribution of *KiSS-1* and *hOT7T175* transcripts in normal and tumour tissues using a quantitative PCR with reverse transcription (RT-PCR) method. Very high *KiSS-1* gene expression was observed in placenta, with the next highest level in testis and moderate levels in pancreas, liver and small intestine (Fig. 5). For *hOT7T175* transcripts, the highest expression levels were found in pancreas and placenta, with moderate expression in spleen, the peripheral blood leukocyte (PBL), testis and lymph node. In the matched complementary DNA pairs that included cDNAs prepared from tumour and normal adjacent tissues of individual patients, tumour samples from four patients had *hOT7T175* expression levels as high as the highest levels observed in pancreas and placenta, whereas normal tissues had low to

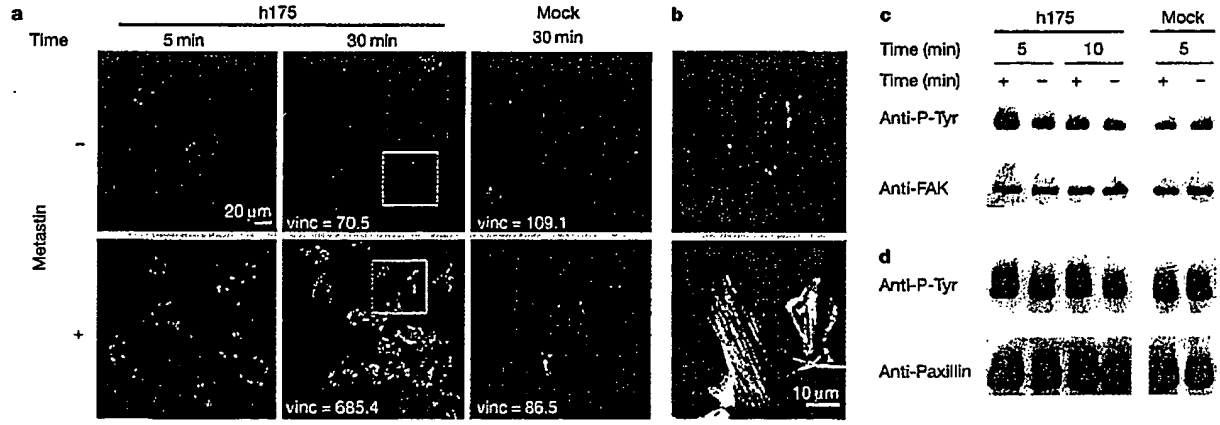

Figure 4 Formation of focal adhesions and stress fibres. B16-BL6/h175 (h175) or B16-BL6/mock melanomas (mock) were challenged with 10 μM metastin (+) or vehicle (–) for indicated period. **a**, Staining with phalloidin (red) and anti-vinculin antibody (green). Vinculin-stained area per single cell is shown in average of arbitrary units (vinc). **b**, High power enlargement of phallodin-stained image from insets in **a**. **c**, **d**, Immunoprecipitate with anti-FAK (**c**) or anti-paxillin antibody (**d**) was analysed by western blotting with indicated antibody.

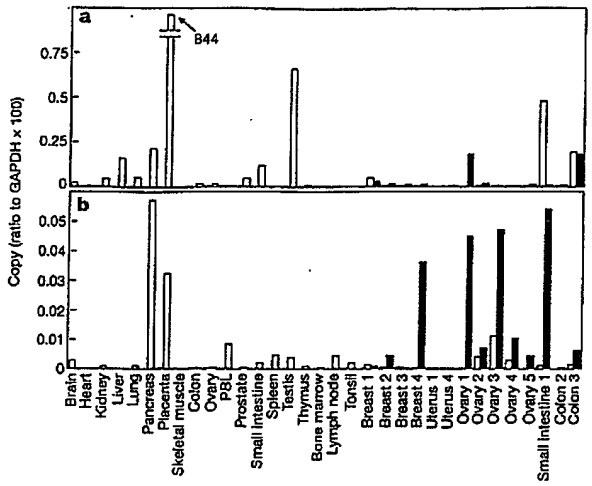

Figure 5 Quantitative RT-PCR analyses of *KiSS-1* and *hOT7T175* transcripts in human multiple tissue cDNA panels and matched cDNA pairs (Clontech). White bars, normal tissues; black bars, tumour tissues. a, *KiSS-1*; b, *hOT7T175*.

negligible levels of expression (Fig. 5). By contrast, high expression of *KiSS-1* gene was not found often in these tumours, but was found in normal tissues of two patients. From these observations, we infer that some human cancers express hOT7T175 but are deficient in metastin. These cancer tissues are potential clinical targets of metastin. It is however unclear whether metastin is physiologically involved in the regulation of cell motility. The best characterized regulation is found in placenta, where the invasive behaviour of cytotrophoblasts is spatially and temporally regulated by metalloproteases, cytokines and growth factors[14]. Considering the abundance of metastin in human placenta, possible involvement of metastin in the regulation of cytotrophoblasts should be investigated in future studies.

The *KiSS-1* gene encodes metastin, the endogenous peptide ligand of the GPCR hOT7T175. Metastin inhibits the chemotaxis, invasion and metastasis of hOT7T175-expressing cells. We propose that the antimetastatic effect of the *KiSS-1* gene may be mediated by metastin and its receptor. It should be investigated whether C8161 melanoma and MDA-MB-435 breast carcinoma used in studies elsewhere[1–3] express hOT7T175 receptor, and whether *KiSS-1* gene-transfected tumour cells secrete metastin. Further studies on the physiological roles and clinical potential of this peptide shall follow. □

Methods

Cloning of *hOT7T175* cDNA

A large part of *hOT7T175* sequence was found in the KAZUSA Genomic DNA Database (VTS35556). The full-length *hOT7T175* cDNA was cloned from a brain cDNA library using a GeneTrapper system (Life Technologies) with biotinylated probes 5′-ATGCACA CCGTGGCTACGTCCG-3′ and 5′-GACCGTGACCAACTTCTACATCGCA-3′. hOT7T175 was stably expressed in CHO/*dhfr*-cells using pAKKO-1.11H vector[15] and in B16-BL6 melanomas using pME18S/neo vector.

Isolation of metastin

The acid extract[7] from human placenta (740 g, Katayama Chemical) was fractionated using a TSKgel ODS80-Tm column (Tosoh, 21.5 × 300 mm)[7] after the protein precipitation, ether extraction and preparative C18 (Waters, 200 ml) chromatography[7]. Active fractions screened by monitoring $[Ca^{2+}]_i$ response (FLIPR assays[16]) were subjected to SP-Sephadex C-25 (0.8 × 3 cm, elution: 2 M pyridine acetate) and Sephadex G50 (1.4 × 80 cm) chromatography[7]. The active peptide was further purified with TSKgel CM-2SW (4.6 × 250 mm, 10–500 mM $HCOONH_4$ gradient in 10% CH_3CN for 60 min at 1 ml min^{-1}), TSKgel Super-Phenyl (4.6 × 100 mm, 21–27% CH_3CN gradient in 0.1% TFA for 60 min at 1 ml min^{-1}), and TSKgel Super-ODS (4.6 × 100 mm, 27–39% CH_3CN gradient in 0.1% HFBA for 60 min at 1 ml min^{-1}) columns.

Receptor binding assay

Assays were done as described[7]. Membrane fraction was prepared using homogenizing buffer (10 mM $NaHCO_3$, 2 mM EGTA, 0.2 mM $MgCl_2$ protease inhibitors, pH 7.4) and stored in 50% glycerol–50% homogenizing buffer at −20 °C. Metastin (40–54) was labelled with ^{125}I-Na using lactoperoxidase, and purified to a carrier-free single peak.

Cell migration and Matrigel invasion assays

A polyvinylpyrrolidone-free polycarbonate framed filter (5-μm pores coated with 10 μg ml^{-1} bovine fibronectin for CHO, 8-μm pores coated with 100 μg ml^{-1} bovine gelatin for B16-BL6 melanomas) was set in a microchemotaxis chamber (Neuro Probe). Cells (2 × 10^5 cells) and peptide were added to the upper chamber and incubated at 37 °C for 6 h (or 7 h for B16-BL6 melanomas) to allow migration to the lower chamber, which contained 10% FCS/DMEM–0.5% BSA (or 5 mg ml^{-1} bovine fibronectin/RPMI 1640–0.5% BSA for B16-BL6 melanomas). After removing cells from the upper surface of the filter, cells on the lower surface were fixed, stained with Diff-Quick (International Reagent) and counted by measuring A_{570}.

Cells and peptide (5 × 10^5 cells per 200 μl DMEM–0.5% BSA) were added to a Matrigel (10 μg per well, Collaborative Res.)-coated Transwell (5-μm pores, Costar) and incubated at 37 °C for 6 h versus a lower chamber containing 10% FCS/DMEM–0.5% BSA. After removing the Matrigel and cells from the upper surface of the membrane, cells on the lower surface were fixed, stained with haematoxylin and eosin, and counted in 0.25-mm^2 fields.

Visualization of focal adhesions and stress fibres

Melanomas (1.5 × 10^5 cells) were plated in collagen IV (Gibco-BRL)-coated chamber slides (Falcon), incubated in RPMI 1640 suspension medium at 37 °C for 2 h, challenged with metastin at 37 °C and then fixed. After washing with 0.3% Triton X-100 PBS (TPBS) and blocking with 10% goat serum/TPBS (STPBS), cells were incubated with anti-vinculin antibody (Sigma) at 4 °C overnight, washed with TPBS and stained with FITC-conjugated anti-mouse IgG (Jackson) at room temperature for 2 h. Cells were further washed with TPBS, stained with rhodamine phalloidin (Molecular Probe) at room temperature for 20 min and washed with TPBS. Cells were mounted with VECTASHIELD and examined under a Leica TCS II confocal microscope.

Phosphorylation of FAK and paxillin

Melanomas (4 × 10^6 cells in RPMI 1640) were plated onto collagen-IV-coated 6-cm dishes, incubated at 37 °C for 2 h, challenged with metastin at 37 °C and lysed with 1 ml of lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EGTA, 2 mM Na_3VO_4, 50 mM NaF, 1% NP-40, 4 mM $Na_4P_2O_7$ and protease inhibitors, pH 7.4). Each 0.45-ml lysate was precipitated with anti-FAK (Upstate) or anti-paxillin (Zymed Laboratory) antibody using protein G-Sepharose. One half of the precipitate was western blotted using ECL phosphorylation detection (Amersham Pharmacia), and the other half was western blotted using anti-FAK or anti-paxillin antibody.

Spontaneous and experimental tumour metastasis assays

Melanomas (3 × 10^5 cells per 20 μl) were injected into the forefoot fat pad of female C57 BL/6 6-week-old mice on d0. On d18, an Alzet pump (duration, 14 days, pumping rate, 0.25 μl h^{-1}, Model 1002, Alza) containing 1 mM metastin (100 μl in distilled water) was implanted subcutaneously. On d21, tumours were surgically removed. On d35, the mice were killed and the lungs were fixed with Bouin's fixative. Tumour foci were counted macroscopically. For experimental metastasis assays, an Alzet pump was implanted three days before cells (5 × 10^4 cells per 100 μl) were injected through the tail vein on d0. The mice were killed on d14.

Quantitative RT-PCR analyses

Analyses were made using a Prism 7700 sequence Detector (PE Biosystems). Primers and fluorescence-labelled probes were: 5′-ACTCACTGGTTTCTTGGCAGC-3′, 5′-ACCTTTTCTAATGGCTCCCCA-3′ and 5′-Fam–ACTGCTTTCCTCTGTGCCACCC ACT–Tamra-3′ for *KiSS-1*, or 5′-CGACTTCATGTGCAAGTTCGTC-3′, 5′-CACACTCA TGGCGGTCAGAG-3′ and 5′-Fam–ACTACATCCAGCAGGTCTCGGTGCAGG–Tamra-3′ for *hOT7T175*.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Correspondence and requests for materials should be addressed to T.O. (e-mail: Ohtaki_Tetsuya@takeda.co.jp). The nucleotide sequence data reported here will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB051065 (*hOT7175*) and AB051066 (*rOT7T175*).

erratum

Self-assembly of mesoscopically ordered chromatic polydiacetylene/silica nanocomposites

Yunfeng Lu, Yi Yang, Alan Sellinger, Mengcheng Lu, Jinman Huang, Hongyou Fan, Raid Haddad, Gabriel Lopez, Alan R. Burns, Darryl Y. Sasaki, John Shelnutt & C. Jeffrey Brinker

Nature 410, 913–917 (2001)

A symbol was omitted from the author list. Yi Yang should have been denoted as contributing equally to this work with Yunfeng Lu. □

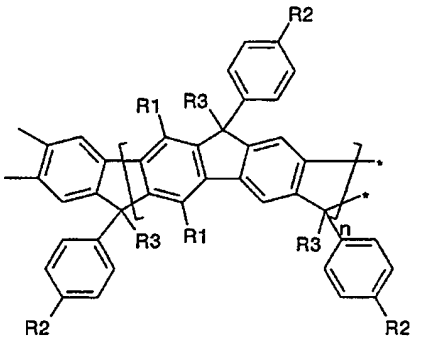
corrections

Formation cross-sections of singlet and triplet excitons in π-conjugated polymers

M. Wohlgenannt, Kunj Tandon, S. Mazumdar, S. Ramasesha & Z. V. Vardeny

Nature 409, 494–497 (2001)

The molecular structure of the π-conjugated polymer mLPPP shown as an inset to Fig. 1a is incorrect; the correct structure is shown below. Furthermore, we regret not to have acknowledged U. Scherf and K. Muellen, who synthesized the mLPPP polymer. □



mLPPP (R1 = n-C_6H_{13}, R2 = n-$C_{10}H_{21}$ R3 = CH_3)

Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses

Carin C. Stamper, Yan Zhang, James F. Tobin, David V. Erbe, Shinji Ikemizu, Simon J. Davis, Mark L. Stahl, Jasbir Seehra, William S. Somers & Lidia Mosyak

Nature 410, 608–611 (2001)

The Protein Data Bank accession number given was incorrect. The correct accession number is 1I8L. □

Reference B (2.)

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Metastin and its variant forms suppress migration of pancreatic cancer cells

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Abstract

Metastin, a post-translationally modified variant of KiSS1, was recently identified as an endogenous peptide agonist for a novel G-protein coupled receptor, hOT7T175 (AXOR12, GPR54). In this study, we analyzed the role of KiSS1 and hOT7T175 in both pancreatic cancer tissues and pancreatic cancer cell lines. Furthermore, we synthesized novel short variant forms of metastin and tested the inhibitory effect of those variants on in vitro cell functions that are relevant to metastasis. Pancreatic cancer tissues showed significantly lower expression of KiSS1 mRNA than normal tissues ($p = 0.018$), while cancer tissues showed significantly higher expression of hOT7T175 mRNA than normal pancreatic tissues ($p = 0.027$). In human pancreatic cancer cell lines, KiSS1 mRNA was highly expressed in 2 out of 6 pancreatic cancer cell lines, while hOT7T175 mRNA was expressed in all cell lines at various degrees. PANC-1 cells showed the highest expression of hOT7T175. Exogenous metastin did not suppress cell proliferation but significantly reduced the in vitro migration of PANC-1 cells ($p < 0.01$). Metastin induced activation of ERK1 in PANC-1 and AsPC-1 cells. Finally, we synthesized 3 novel short variant forms of metastin, FM053a2TFA, FM059a2TFA, and FM052a4TFA. These metastin variants significantly suppressed the migration of PANC-1 cells and activated ERK1. These data suggest that the metastin receptor, hOT7T175, is one of the promising targets for suppression of metastasis, and that small metastin variants could be an anti-metastatic agent to pancreatic cancer.

Keywords: KiSS1; hOT7T175; Metastin; Pancreatic cancer; Migration; Metastasis

The KiSS1 peptide was originally identified as being differentially up-regulated in C8161 melanoma cells that have been rendered to have non-metastatic function by microcell mediated transfer of human chromosome 6 [1]. Transfection of *KiSS1* into human melanoma and breast carcinoma cells prevents these cells from metastasizing without an effect on cell proliferation [2]. Furthermore, the KiSS1 product has been shown to repress 92-kDa type 4 collagenase (MMP-9) expression by affecting NF-κB binding to the promoter [3]. The KiSS1 product was found to be expressed in normal human placenta, testis, brain, pancreas, and liver [4].

Recently, it was shown that the human metastasis suppressor gene *KiSS1* encodes a COOH-terminally amidated peptide with 54 amino acid residues, and that this peptide is a ligand of a novel human G-protein coupled receptor (AXOR12 and hOT7T175) which couples primarily to Gq/11 [4–7]. The receptor has a high degree of homology (81% amino acid identity) to the rat orphan heptahelical receptor, GPR54 [8], indicating that these two receptors are orthologs. The peptide ligand, named as metastin, enhances the expression and activity of focal adhesion kinase, and attenuates pulmonary metastasis of hOT7T175 transfected

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B16-BL6 melanomas in vivo [5]. In another experiment, metastin inhibits chemotaxis and invasion of hOT7T175 transfected Chinese hamster ovary cells (CHO cells) in vitro with the activation of phospholipase C, arachidonic acid release, and phosphorylation of ERK [6,7]. These indicate that metastin-hOT7T175 axis may act as an anti-metastatic system. The characteristics of inhibitory effects on cancer cell metastasis without affecting cellular growth properties of normal cells make the metastin receptor to be an attractive target for cancer therapy.

The *KiSS1* is located on human chromosome1q32–q41 [9]. However, evidences from subsequent experiments suggest that the expression of *KiSS1* is regulated by a gene(s) located in the region between 6q16.3 and q23 [1]. In pancreatic cancer, losses of 6q, 8p, 9p, 17p, and 18q are frequently observed and those alterations tend to cause lymph node and distant metastases, which suggests a suppressor gene(s) important for pancreatic cancer metastasis may exist in these regions [10–12]. Therefore, pancreatic cancer, has good reasons to downregulate KiSS1 expression. Moreover, in other cancers such as ovarian cancer, breast cancer, and thyroid papillary cancer, over-expression of hOT7T175 has been demonstrated, although KiSS1 is less frequently expressed in the tumor tissue [4,5]. Until now, however, expressions of KiSS1 and hOT7T175, and their function have not been investigated in pancreatic cancer. The purpose of the present study was to determine if KiSS1 and its receptor hOT7T175 are expressed in pancreatic cancer tissues, and to analyze the effect of exogenous metastin on pancreatic cancer cell lines with different expression levels of hOT7T175. Finally, we newly synthesized short variant forms of metastin and tested the inhibitory effect of those variants on in vitro cell functions that are relevant to metastasis.

Materials and methods

Cell culture. Pancreatic cancer cell lines, AsPC-1, BxPC-3, Capan-2, CFPAC-1, PANC-1, and SUIT-2 were purchased from the American Type Culture Collection. Cells were cultured as monolayers in the appropriate medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humid atmosphere of 5% CO_2/95% air. As for AsPC-1 and PANC-1, upon reaching 80% confluence, the medium was removed, the cells were washed in phosphate-buffered saline (PBS) and treated with various concentrations of metastin (Takeda Chemical Industries, Tsukuba, Japan) with 10% fetal bovine serum, and protein was isolated 15 min later as described below.

Patients and tumor samples. Pancreatic cancer tissues obtained from 30 patients who underwent pancreatectomy at our Department between January 1998 and June 2001 were used. Patients with other pancreatic malignancies, such as intraductal papillary mucinous adenocarcinoma, acinar cell carcinoma, and endocrine tumor, were excluded. Informed consent was obtained from each patient according to the institutional guidelines. Samples for mRNA expression were immediately frozen in liquid nitrogen at the time of surgery and stored at −80 °C.

Peptide synthesis. Fifty-four amino acid peptide, metastin, was kindly provided from Dr. T. Ohtaki, Takeda Chemical Industries [5]. We synthesized 3 short peptide variants of metastin, which were defined as FM053a2TFA, FM059a2TFA, and FM052a4TFA. The sequences of these peptides are as follows:

FM053a2TFA: Gu-Amb-Phe-Gly-Leu-Arg-Trp-NH_2,
FM059a2TFA: Ac-Trp-Asn-Arg-Phe-Gly-Leu-Arg-Trp-NH_2,
FM052a4TFA: Bis(Py)-Amb-Phe-Gly-Leu-Arg-Trp-NH_2.

These peptides were selected by screening from various truncated forms of KiSS1 peptide and modified peptides for gaining almost the same internal signals with metastin through the receptor using reporter gene assay in yeast.

All reagents for peptide synthesis were purchased from Watanabe Chemical Industries (Hiroshima, Japan), NovaBiochem (Darmstadt, Germany), Nacalai Tesque (Kyoto, Japan), and Wako Pure Chemical Industries (Osaka, Japan). Ion-spray mass spectra were obtained with a Sciex APIIIIE triple quadrupole mass spectrometer. Protected peptide-resins were manually constructed on Fmoc-NH-SAL resin by Fmoc-based solid-phase peptide synthesis. Trt for Asn and Pbf for Arg were employed for side-chain protection. In the synthesis of FM052a and FM053a, amino-group modifications were performed after coupling of 4-(aminomethyl)benzoic acid, respectively. Reductive amination using pyridine-2-aldehyde and $NaBH_3$ (OAc) provided the protected resin for FM052a. Treatment with 1H-pyrazole-1-carboxamidine hydrochloride and *N*,*N*-diisopropylethylamine gave the resin for FM053a. Deprotection/cleavage by treatment of the peptide resins with a mixture of TFA-thioanisole-*m*-cresol-H_2O-1,2-ethanedithiol-triisopropylsilane (80:5:5:5:2.5:2.5, v/v) followed by purification by reverse phase HPLC (Cosmosil 5C18-ARII column, Nacalai Tesque, Japan, 20 × 250 mm) yielded the peptides. FM059a was prepared by N-terminal acetylation of the protected octapeptide, subsequent deprotection/cleavage, and HPLC purification as well.

Quantitative RT-PCR. To monitor gene expression, we used quantitative real-time RT-PCR analysis [13–15]. Briefly, within the amplicon defined by a gene-specific PCR primer pair, an oligonucleotide probe labeled with 2 fluorescent dyes is created and designated as TaqMan probe. As long as the probe is intact, the emission of the reporter dye (6-carboxy-fluorescein, FAM) at the 5′-end is quenched by the second fluorescence dye (6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3′-end. During the extension phase of PCR, the polymerase cleaves the TaqMan probe, resulting in a release of the reporter dye. The increasing amount of reporter dye emission is detected by an automated sequence detector combined with analysis software (ABI Prism 7700 Sequence Detection System; PE Applied Biosystems). The conditions of the reaction were according to the manufacturer's protocol. Five microliters of cDNA (reverse transcription mixture) with 25 μl TaqMan Universal PCR Master Mix (PE Applied Biosystems) and oligonucleotides at a final concentration of 0.3 μM for primers and 0.2 μM for the TaqMan hybridization probe were analyzed in a 50-μl volume.

The following primers and TaqMan probes were used for analysis. The KiSS1 specific primers were

5′-ACTCACTGGTTTCTTGGCAGC-3′ (upstream primer),
5′-ACCTTTTCTAATGGCTCCCCA-3′ (downstream primer), and
5′ (FAM)-ACTGCTTTCCTCTGTGCCACCCACT-(TAMRA)3′ (TaqMan probe).

The hOT7T175 specific primers were

5′-CGACTTCATGTGCAAGTTCGTC-3′ (upstream primer),
5′-CACACTCATGGCGGTCAGAG-3′ (downstream primer), and
5′ (FAM)-ACTACATCCAGCAGGTCTCGGTGCAGG-(TAMRA)3′ (TaqMan probe).

The thermal cycle parameters were 95 °C for 10 min (for heat activation of *Taq*-Polymerase), followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Assessment of GAPDH RNA for quality and normalization was done with the TaqMan GAPDH Control Reagent Kit (PE Applied Biosystems) which utilizes standard TaqMan probe chemistry.

Protein extraction and Western blotting. Cells were collected into microtubes with a cell scraper and lysed for 60 min in phosphorylation-inhibitory RIPA buffer containing 50 mM Hepes (pH 7.0), 250 mM NaCl, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 μg/ml gabexate mesilate, and then the lysate was sonicated for 10 s. Total extracts were cleaned by centrifugation at 12,000 rpm for 10 min at 4 °C and the supernatants were collected. Protein concentrations were measured using a protein assay kit (Tonein-TP, Otsuka Pharmaceutical, Tokyo, Japan). The lysates were resuspended in one volume of the gel loading buffer which contained 50 mM Tris–HCl (pH 6.7), 4% SDS, 0.02% bromophenol blue, 20% glycerol and 4% 2-mercaptoethanol, and then boiled at 95 °C for 90 s. The extracted protein was subjected to Western blotting, as previously described [16]. In brief, 30-μg aliquots of protein were size-fractionated to a single dimension by SDS–PAGE (12% gels) and transblotted to a 0.45-μm polyvinylidene difluoride membrane (Bio-Rad, Richmond, CA) in a semidry electroblot apparatus (Bio-Rad, Richmond, CA). The blots were then washed 3 times with TBST buffer and incubated for 2 h at RT in the first antibody solution containing anti-phospho-ERK antibody (pTEpY, Promega, Madison, WI), anti-phosphop38 MAPK (pTGpY, Promega, Madison, WI) or anti-phosphoJNK (pTPpY, Promega, Madison, WI), 0.2% I-block (Promega, Madison, WI). After 3 washings in TBST buffer, the blots were incubated for 1 h at RT with horseradish peroxidase-conjugated anti-rabbit IgG at a 1:2000 dilution with TBST buffer. After 3 washings in TBST buffer, membranes were treated with enhanced chemiluminescence reagents (Amersham Life Sciences, Amersham, UK) according to the manufacturer's protocol. Membranes were exposed to X-ray film for 50–60 s. Protein expression was measured by ATTO densito-analyzer system AE-6920M (ATTO Corporation, Tokyo Japan) and the quantity was expressed numerically. The quantity of the target protein was divided by that of β-actin and relative intensities were calculated.

Cell proliferation assay. AsPC-1 and PANC-1 cells (1×10^4 cells/3 cm diameter dish) were seeded in 10% FBS medium and incubated with increasing doses of metastin for 48 and 96 h. Cells were trypsinized and cell numbers were counted using hematocytometer.

Cell migration assay and Matrigel invasion assays. A polyvinyl-pyrrolidone-free polycarbonate framed filter (8 μm pores) was set in a chamber (Corning Coster, Cambridge, MA). Cells (2×10^6 cells in 200 μl RPMI1640 for AsPC-1 and in 200 μl DMEM for PANC-1) and designated concentrations of peptide were added to the upper chamber and incubated at 37 °C for 12 h to allow migration to the lower chamber, which contained 10% FBS/RPMI1640 for AsPC-1 or 5% FBS/DMEM for PANC-1 as a chemoattractant. After removing non-migrating cells with a cotton swab from the upper surface of the membrane, cells on the lower surface were fixed, stained with Diff-Quick (International Reagent, Kobe, Japan). For quantification, cells were counted under a microscope in 5 predetermined fields at 200×.

Cells and peptide (2×10^6 cells in 200 μl RPMI1640 for AsPC-1 and in 200 μl DMEM for PANC-1) were added to a Matrigel-coated Transwell (8 μm pores, Becton–Dickinson Labware, Bedford, MA) and incubated at 37 °C for 12 h versus a lower chamber containing 10% FBS/RPMI1640 for AsPC-1 or 5% FBS/DMEM for PANC-1. After removing the Matrigel and cells from the upper surface of the membrane, cells on the lower surface were fixed, stained with Diff-Quick and the number quantified as well. Invasion index was defined as the number of invaded cells per that of migrated cells.

Statistical analyses. The comparative statistical evaluations among groups in the densitometry or in the migratory activity were first performed by a two-way analysis of variance for repeated measures, followed by a post hoc Tukey test. To compare the mRNA levels in pancreatic tissues, Wilcoxon's rank sum test was performed. All assays were performed 3 times independently. Statistical analyses were done using JMP statistical software (version 3.02). Probability value of <0.05 was considered significant.

Results

Expression of KiSS1 and hOT7T175 in pancreatic cancer tissues

First, we measured the mRNA expression levels of KiSS1 and hOT7T175 in 30 pancreatic cancer tissues and in 5 adjacent normal pancreatic tissues. All the normal pancreatic tissues (5/5) and 14/30 of pancreatic ductal carcinoma tissues expressed KiSS1 mRNA (Fig. 1A). The expression level of KiSS1 mRNA in

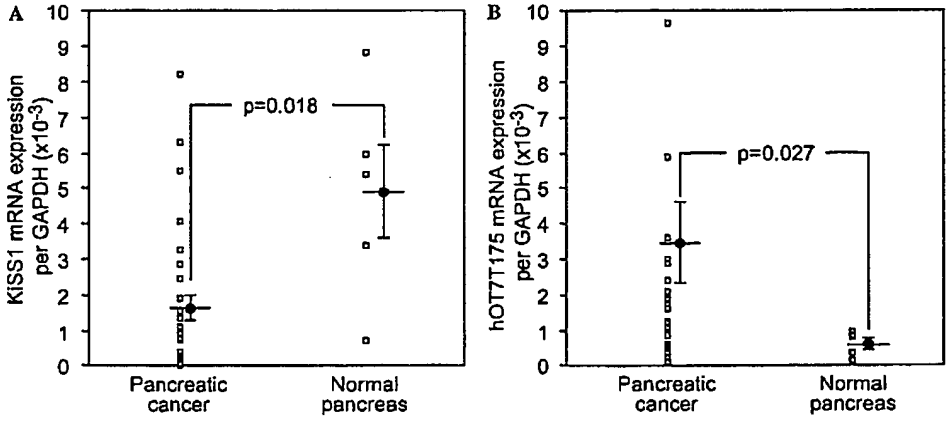

Fig. 1. Expression of KiSS1 mRNA and hOT7T175 mRNA in pancreatic cancer tissues. The KiSS1 mRNA and hOT7T175 mRNA in pancreatic cancer ($n = 30$) and normal pancreatic tissues ($n = 5$) were measured by real-time RT-PCR. The level of KiSS1 mRNA in pancreatic cancer tissues was significantly lower than that of normal pancreatic tissues ($p = 0.018$). The level of hOT7T175 mRNA in pancreatic cancer tissues was significantly higher than that of normal pancreatic tissues ($p = 0.027$).

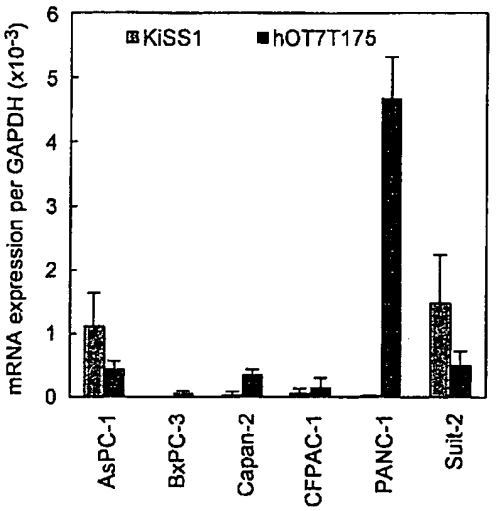

Fig. 2. Expression of KiSS1 mRNA and hOT7T175 mRNA in pancreatic cancer cell lines. The KiSS1 mRNA and hOT7T175 mRNA in pancreatic cancer cells were measured by real-time RT-PCR. AsPC-1 and SUIT-2 showed high level of metastin mRNA expression and other 4 cell lines showed very low level of expression. In contrast, all cell lines expressed hOT7T175 mRNA. PANC-1 cell most highly expressed hOT7T175 mRNA. Measurements were repeated three times and data are expressed as means ± SEM.

pancreatic cancer tissues was significantly lower than normal pancreatic tissues ($p = 0.018$). In contrast, all the pancreatic cancer tissues (30/30) and normal pancreatic tissues (5/5) expressed KiSS1 receptor hOT7T175 mRNA, and the expression level of hOT7T175 mRNA in pancreatic cancer tissues was significantly higher than that of normal tissues ($p = 0.027$) (Fig. 1B). We tested paired samples from 5 patients (cancer and normal tissues from each patient). The expression of hOT7T175 was higher in cancer tissue than the adjacent normal pancreatic tissue in all the 5 paired samples.

Expression of KiSS1 and hOT7T175 in pancreatic cancer cell lines

We next measured the expression of KiSS1 mRNA and hOT7T175 mRNA in 6 pancreatic cancer cell lines (Fig. 2). Among 6 pancreatic cancer cell lines, AsPC-1 and SUIT-2 showed high level of KiSS1 mRNA expression and the other 4 cell lines showed a very low level of expression. In contrast, all cell lines expressed hOT7T175 mRNA at various degrees. PANC-1 cell most strongly expressed hOT7T175 mRNA. According to these results, we chose AsPC-1 cell line as a representative of high KiSS1 and low hOT7T175, and chose PANC-1 cell line as that of low KiSS1 and high hOT7T175. We used these two cell lines in the following experiments.

Effects of exogenous metastin on proliferation, migration, and invasion through endogenous metastin receptor

We examined the effect of exogenous metastin on pancreatic cancer cell proliferation. Metastin peptide was added to AsPC-1 and PANC-1 cells in the phase of exponential growth at final concentrations of 0, 0.1, 1, and 10 μM for 48 and 96 h. The addition of metastin had no effects on cell proliferation of AsPC-1 and PANC-1.

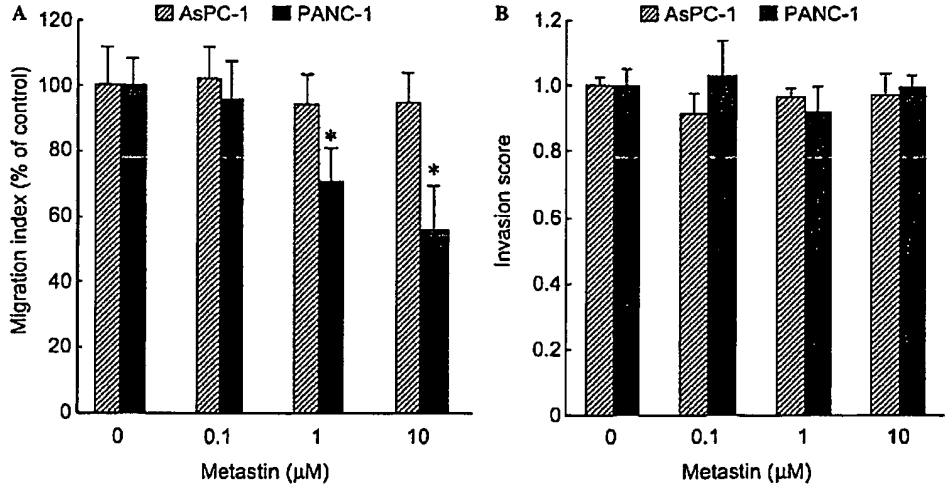

Fig. 3. Effects of exogenous metastin on migration and invasion of pancreatic cancer cells. (A) Effect of metastin on migration of pancreatic cancer cells. Cells were treated with various concentrations of metastin for 12 h. Metastin at 1 and 10 μM significantly suppressed migratory activity of PANC-1 cells. (B) Effect of metastin on invasion of pancreatic cancer cells. Cells were treated with various concentrations of metastin for 12 h. Invasion activity was not affected by tested concentrations of metastin in both AsPC-1 and PANC-1. Experiments were repeated three times and data are expressed as means ± SEM. * Represents $p < 0.05$ against control.

Next, we tested the effect of metastin on migration and invasion of these cell lines. The migration of AsPC-1 was not significantly affected by metastin, while PANC-1 was significantly inhibited by metastin at 1 and 10 μM ($p < 0.05$) (Fig. 3A). The invasion of the two cell lines was not significantly affected by metastin (Fig. 3B).

Effects of metastin on MAPK activation in AsPC-1 and PANC-1 cells

We analyzed the activation of MAPK by metastin. Cancer cells in exponential phase were incubated in serum containing medium and then transferred to 1% BSA medium with metastin as described in the section of migration and invasion assay. After incubation with metastin for 15 min, ERK1/2, p38, and JNK1/2 were investigated by immunoblotting (Fig. 4). Protein expression was measured by ATTO densito-analyzer system AE-6920M (ATTO Corporation, Tokyo Japan) and the relative intensity was expressed numerically (Fig. 5). Metastin induced a significant increase of pERK1 in AsPC-1 cells at 1 and10 μM and in PANC-1 at 0.1–10 μM (Fig. 5A). Metastin induced a significant increase of pp38 in PANC-1 cells at 10 μM (Fig. 5B).

Effects of short variant forms of metastin on proliferation and migration of hOT7T175 expressing pancreatic cancer cells

We analyzed the effects of metastin and newly synthesized short peptides, FM053a2TFA, FM059a2TFA, and FM052a4TFA, on PANC-1 cells which highly express hOT7T175. We found that the cell growth was not affected by these 3 peptides. In migration assay, metastin, FM059a2TFA, and FM052a4TFA significantly inhibited the migration of PANC-1 cells (Fig. 6). We examined the activation of ERK1/2, p38, and JNK by these variant forms and found that ERK1 and p38 were activated by metastin and all variant forms of metastin (Fig. 7).

Discussion

In this study, we have demonstrated for the first time that hOT7T175 is expressed in pancreatic cancer tissues, but KiSS1 is less expressed when compared to normal pancreatic tissues. These results are in agreement with the analysis by other investigators in ovarian cancer, breast cancer, and colon cancer [2,5]. Several reports indicated that KiSS1 and its receptor hOT7T175 are also highly expressed in placenta [4,5]. The placenta is an invasive tissue, and there are similarities in the behavior of invading cancer cells and that of invading placenta cells [17]. It is possible that KiSS1 and hOT7T175 may constitute a common mechanism in both of these processes, whereas the correlations of clinicopathological factors such as distant metastasis and invasion with KiSS1/hOT7T175 function have not been clearly proved yet.

We next demonstrated that PANC-1 cells, which express hOT7T175, showed significant suppression of cell migration with concomitant activation of ERK1 but not

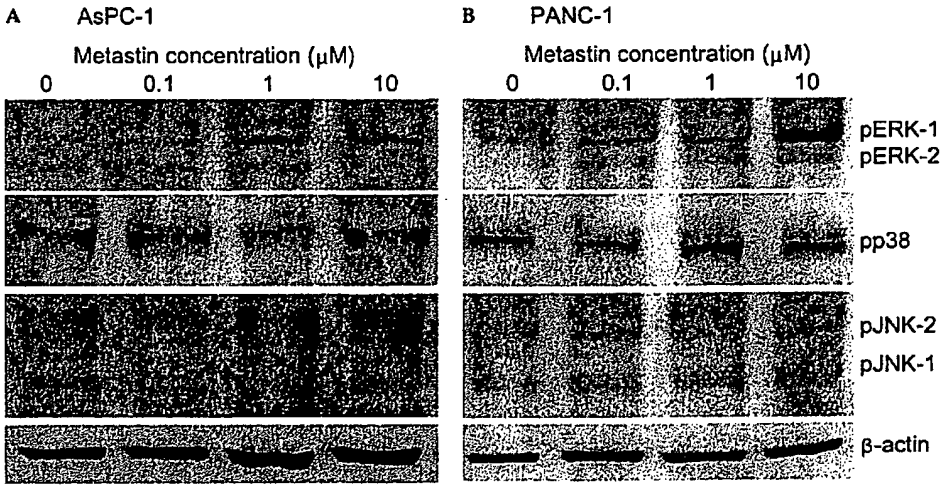

Fig. 4. Effects of metastin on MAPK activation in AsPC-1 and PANC-1 cells. AsPC-1 and PANC-1 cells were treated with various concentrations of metastin for 15 min. Western blot analysis identified double band corresponding to phosphorylated ERK1 (pERK1) and phosphorylated ERK2 (pERK2), single band of phosphorylated p38 (pp38), and double band showing phosphorylated JNK1 (pJNK1) and phosphorylated JNK2 (pJNK2). pERK1 was augmented in AsPC-1 by metastin at 1 and 10 μM and in PANC-1 by metastin at 0.1, 1, and 10 μM. p38 was augmented in PANC-1 by metastin at 10 μM.

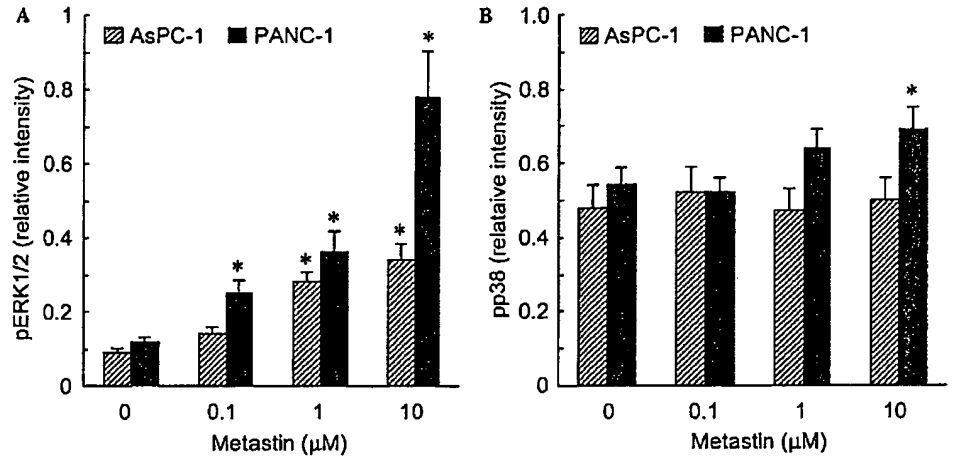

Fig. 5. Effects of metastin on pERK1/2 and of pp38 in AsPC-1 and in PANC-1 cells. AsPC-1 and PANC-1 cells were treated with various concentrations of metastin for 15 min. Protein expression of pERK1/2 or pp38 was measured by a densito-analyzer system and the quantity was expressed numerically. The quantity of the protein was divided by that of β-actin and the relative intensities were calculated. Metastin induced a significant increase of pERK1 in AsPC-1 cells at 1 and 10 μM and in PANC-1 at 0.1–10 μM (A). Metastin induced a significant increase of pp38 in PANC-1 cells at 10 μM (B). Experiments were repeated three times and data are expressed as means ± SEM. * Represents $p < 0.05$ against control.

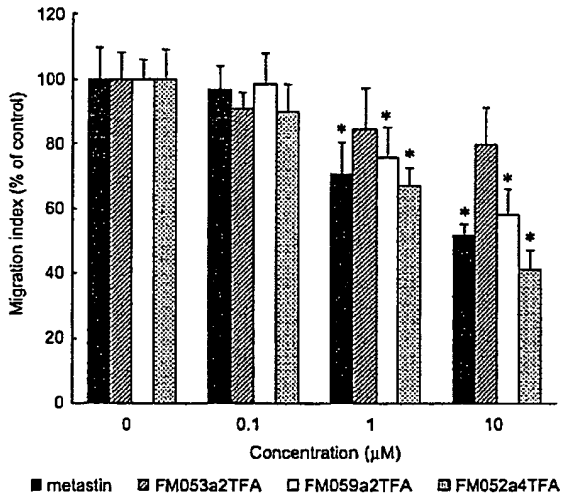

Fig. 6. Effects of short variant forms of metastin on migration of hOT7T175 expressing pancreatic cancer cells. The effect of newly synthesized short variant forms of metastin on migration of PANC-1 cells was evaluated. Metastin, FM059a2TFA, and FM052a4TFA significantly inhibited the migration activity of PANC-1 cells. Experiments were repeated three times and data are expressed as means ± SEM. * Represents $p < 0.05$ against control.

of JNK1/2 in response to exogenous metastin, while AsPC-1 cells with low expression of hOT7T175 revealed comparatively less response. Of note, the cell growth suppression was not observed both in PANC-1 and in AsPC-1, that is consistent with the previous results in melanoma and in breast cancer cells [1,2]. In contrast, in other types of cells, KiSS1 product was reported to activate ERKs and to inhibit cell proliferation [7]. These controversial results suggest that the proliferative characteristics were not a property shared by this receptor. Rather this, ERK activation may be involved in suppression of the tumor cell motility. In our study, suppression of the motility of PANC-1 cells was concomitant with the activation of ERK pathway. Moreover, as demonstrated by the newly synthesized short peptide treatment, the rate of ERK activation is in proportion to the suppression of migration.

Activation of MAP kinase and p38 has been described in hOT7T175 transfected Chinese hamster ovary cells (CHO cells) [7]. However, it has been shown that only MAP kinase but not p38 was activated with metastin treatment in anaplastic thyroid cancer cells ARO, which endogenously express hOT7T175 [18]. This discrepancy may be partly accounted by the expression level of hOT7T175 and may be by the cell specificity. In our experiment, PANC-1 cells with high expression of hOT7T175 showed activation of ERK1 and p38, while AsPC-1 cells with less hOT7T175 expression did not. These results may indicate the necessity of strong metastin-hOT7T175 signals on p38 activation in cancer cells.

We found metastin did not suppress invasion of PANC-1 and AsPC-1 cells, although several authors have reported that metastin suppresses invasion of melanoma and breast cancer cells [2,5]. The inhibitory effect on invasion could be explained by the report that showed that KiSS1 represses the invasion of HT1080 cells through decreased type 4 collagenase (MMP-9) expression and downregulation of NF-κB [3]. In pancreatic cancer tissues, we previously reported that active

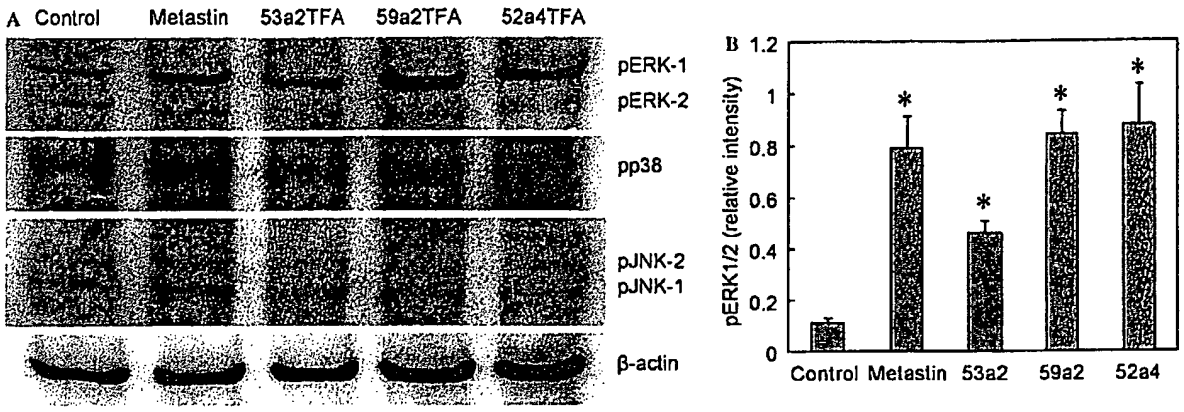

Fig. 7. Effects of short variant forms of metastin on MAPK activation in hOT7T175 expressing pancreatic cancer cells. (A) The activation of ERK1/2, p38, and JNK ERK1 activation by metastin and short variant forms of metastin was observed in PANC-1 cells. (B) pERK1 expression was measured by a densito-analyzer system, the quantity of the protein was divided by that of β-actin, and the relative intensities were calculated. In PANC-1 cells, ERK1 was significantly activated by metastin and all variant forms of metastin. Experiments were repeated three times and data are expressed as means ± SEM. * Represents $p < 0.01$ againstcontrol.

form of MMP-2 was detected in all samples; however, active form of MMP-9 was seen in only 21% of the samples [19]. Because latent forms of MMP-2 and MMP-9 were expressed in all pancreatic cancer tissues, MMP-9 may not mainly contribute to cancer invasion when compared to MMP-2. This might be one of the reasons why metastin did not affect invasion of PANC-1 and AsPC-1 cells. To our knowledge, there has been no other investigation on migration or invasion of cancer cells which endogenously express hOT7T175.

Our newly synthesized short variant forms of metastin showed significant suppression on motility of PANC-1 cells. These peptides are composed of 6–9 amino acids, suggesting that at most 10 amino acids of the C-terminus of metastin-54 will be sufficient for its binding affinity and function. However, FM053a2TFA is less effective in suppressing migration of PANC-1 cells when compared to other two compounds or metastin-54. Interestingly, the suppressive activity was in proportion to the ERK activation rates. Much remains to be understood about how effective they will block migration and how the peptides could be stably delivered to the tumors. The effective short peptide has an advantage that it would not cause immune responses if it could be given to patients orally.

In conclusion, we demonstrated that pancreatic cancer tissues express hOT7T175 and low expression of KiSS1 when compared to normal pancreatic tissues. The exogenous metastin and the variant forms of metastin suppress migration of hOT7T175-expressing pancreatic cancer cells and activate ERK1 and p38. Our results suggest that hOT7T175 may be one of the promising targets against cancer cell functions that are relevant to metastasis, and that short variant forms of metastin could be an anti-metastatic agent to pancreatic cancer.

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Reference C (3)

Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts

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Summary

Trophoblast invasion of the uterine extracellular matrix, a critical process of human implantation and essential for fetal development, is a striking example of controlled invasiveness. To identify molecules that regulate trophoblast invasion, mRNA signatures of trophoblast cells isolated from first trimester (high invasiveness) and term placentae (no/low invasiveness) were compared using U95A GeneChip microarrays yielding 220 invasion/migration-related genes. In this 'invasion cluster', KiSS-1 and its G-protein-coupled receptor KiSS-1R were expressed at higher levels in first trimester trophoblasts than at term of gestation. Receptor and ligand mRNA and protein were localized to the trophoblast compartment. In contrast to KiSS-1, which is only expressed in the villous trophoblast, KiSS-1R was also found in the extravillous trophoblast, suggesting endocrine/paracrine activation mechanisms. The primary translation product of *KiSS-1* is a 145 amino acid polypeptide (Kp-145), but shorter kisspeptins (Kp) with 10, 13, 14 or 54 amino acid residues may be produced. We identified Kp-10, a dekapeptide derived from the primary translation product, in conditioned medium of first trimester human trophoblast. Kp-10, but not other kisspeptins, increased intracellular Ca^{2+} levels in isolated first trimester trophoblasts. Kp-10 inhibited trophoblast migration in an explant as well as transwell assay without affecting proliferation. Suppressed motility was paralleled with suppressed gelatinolytic activity of isolated trophoblasts. These results identifed Kp-10 as a novel paracrine/endocrine regulator in fine-tuning trophoblast invasion generated by the trophoblast itself.

Key words: Invasion, DNA microarray, Kisspeptins, Metastin, Trophoblast

Introduction

The invasion of trophoblast cells (cytotrophoblasts), epithelial cells of the placenta, into the maternal decidua in the first trimester of pregnancy is a key process for successful reproduction and embryonic development. As a result, the embryo will be anchored in the maternal uterus and utero-placental arteries will be invaded, leading to the dilation of the vessel lumina, which is necessary to enhance uterine and intervillous blood flow in pregnancy. There are striking similarities between invasive cytotrophoblasts and invasive cancer cells (Kliman and Feinberg, 1990; Murray and Lessey, 1999; Bischof and Campana, 2000; Bilban et al., 2000; Murray and Lessey, 1999; McMaster et al., 1998). However, in contrast to tumor invasion, interactions between trophoblasts and uterine cells are closely regulated both temporally and spatially by mechanisms that are largely unknown. If mechanisms controlling migration and invasion of both trophoblast and neoplastic cells are related, it is tempting to postulate that genes expressed in early pregnancy, a period necessitating a tight control of these processes, also play a key role in regulating migration and invasion of cancer cells.

KISS-1 is one of the genes involved in controlling cancer cell dissemination. Differential display and subtractive hybridization were used to isolate *KiSS-1* from melanoma cells (Lee et al., 1996). Its expression is lost during cancer progression, and overexpression of KiSS-1 cDNA in metastatic human cancer cell lines suppressed metastasis in athymic nude mice (Lee and Welch, 1997; Shirasaki et al., 2001; Sanchez-Carbayo et al., 2003). The primary translation product of the *KiSS-1* gene is a 145 amino acid polypeptide (Kp-145) (Ohtaki et al., 2001), but shorter 'kisspeptins' (Kp) with 10, 13, 14 or 54 amino acid residues have been discovered (Fig. 1) (Kotani et al., 2001; Stafford et al., 2002; Harms et al., 2003). Kps are endogenous ligands for an orphan G-protein-coupled receptor (GPR54, hOT7T175, AXOR12; KiSS-1R as designated from here on) that couples primarily to Gq/11 (Ohtaki et al., 2001; Kotani et al., 2001; Muir et al., 2001). KiSS-1R and its cognate ligand KiSS-1 are expressed in a variety of tissues including

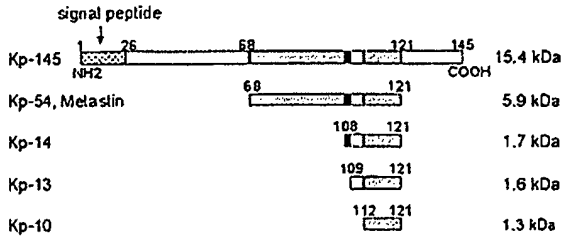

Fig. 1. KiSS-1 sequence and cleavage products resulting in various kisspeptins.

pancreas, testes, central nervous system and placenta (Janneau et al., 2002; Harms et al., 2003). Kp-54, also termed 'metastin', is the putatively secreted and biological active form of Kp-145 (Harms et al., 2003). It inhibits chemotaxis in vitro, enhances the expression and activity of focal adhesion kinase and inhibits melanoma cell metastasis (Ohtaki et al., 2001). Kp-10 stimulates phosphatidylinositol-4,5-bisphosphate hydrolysis, Ca^{2+} mobilization, arachidonic acid release, extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein (MAP) kinase phosphorylation, and stress fiber formation (Kotani et al., 2001). These effects have been found in cells overexpressing KiSS-1R, but have not been investigated in a physiological system so far. Human first trimester (FT) placenta enables the mechanisms of invasion and its regulation to be investigated in a physiological setting.

FT trophoblasts secrete collagenases MMP-2 and MMP-9 to degrade extracellular matrices, thus making migration and invasion possible (Bischof and Campana, 2000; Xu et al., 2001). Interestingly, the KiSS-1 transcript product represses MMP-9 activity (Yan et al., 2001).

Despite their putative importance in invasion regulation in a biological system, neither Kp-10 has been detected nor have the effects of Kps been investigated. Here we report for the first time the identification of Kp-10 as a naturally occurring peptide in conditioned medium of primary human trophoblasts, its ability to reduce trophoblast migration without altering proliferation, and to downregulate the activity of MMP-2, one of the key enzymes in trophoblast invasion.

Materials and Methods

Reagents

Collagen type I (rat tail tendon), fibronectin and Matrigel were from Beckton Dickenson (Oxford, UK), Sigma (St Louis, MO) and Stratech Scientific (Luton, UK), respectively. Kp-10, Kp-13 and Kp-14, corresponding to KiSS-1 aa 112-121, 109-121 and 108-121, respectively (Ohtaki et al., 2001; Kotani et al., 2001), were chemically synthesized. Kp-54 (corresponding to aa 68-121 of KiSS-1) was purchased from California Peptide Research (Nappa, CA). A polyclonal antibody to Kp-54 was raised in rabbits. Polyclonal rabbit anti-KISS-1R (human) antibody (cl375-398) was from Phoenix Pharmaceuticals (Belmont, CA).

Immunization protocol

Two milligrams of Kp-54 (California Peptides) were dissolved in 0.1 M 2-(4-Morpholino)-ethanesulfonic acid buffer (pH 4.9) and coupled to 3 mg KLH (keyhole limpet hemocyanine) by use of a water-soluble derivative of EDCI (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimid-hydrochloride) as a condensation agent (Hermanson, 1996). After 2 hours EDCI and other low molecular weight molecules were removed by size exclusion chromatography (PD-10 columns, Sephadex G25 M, Amersham) using PBS as purification buffer. Two New Zealand White rabbits were immunized subcutaneously by the use of complete Freud Adjuvant and two boosts after 4 weeks and 2 weeks, respectively, with incomplete Freud Adjuvant according to standard protocols used by Charles River (Kisslegg, Germany). Globulins were precipitated from the isolated sera by ammonium sulfate and IgG were purified by protein-A affinity chromatography (Pierce, Rockford, IL).

Tissue and cells

All patients gave informed consent for collection and investigational use of tissues. This study was approved by the ethics committee of the Karl-Franzens University (Graz, Austria). FT placental tissue was obtained after termination of normal pregnancies by vacuum suction; term placentae were collected after uncomplicated pregnancy and vaginal delivery. Tissues were immediately used for experiments or fixed for 48 hours in 20% formalin buffered with 0.1 M phosphate buffer, pH 7.4, embedded in paraffin, and cut into 4 μm (immunohistochemistry) or 7 μm (in situ hybridization) sections. Five different placentae with at least ten sections of each were examined.

Mononucleated trophoblast cells were isolated by tissue digestion with trypsin and subsequent separation of released cells on a density gradient. Cells from the bands containing trophoblasts were further purified using immunomagnetic beads conjugated with either a combination of anti-CD45RB and fibroblast-specific antibodies (FT) or anti-HLA class-I antibodies (term) to remove nontrophoblast components. Isolated cells were cultured in Dulbecco's Eagle's Medium (DMEM; Gibco, Life Technologies, Paisley, UK) and rigorously characterized as described in detail elsewhere (Blaschitz et al., 2000; Cervar et al., 1999).

cRNA synthesis and gene expression profiling

Preparation of cRNA, hybridization to human U95A GeneChips (Affymetrix, Santa Clara, CA) and scanning of the arrays were carried out at The Scripps Research Institute's Affymetrix Array Core facility (http://www.scripps.edu/services/dna_array/) according to the manufacturer's protocols (available online at https://www.affymetrix.com; (Su et al., 2002)). Images were analyzed with GeneChip software (Affymetrix, version 3.3). Genes were scored on the basis of the putative biological functions of the encoded proteins, as determined by database searches on PubMed, gene cards from the Weizmann Institute of Science (http://bioinfo.weizmann.ac.il/bioinfo.html), and a previously published classification scheme ('OntoExpress') for cellular functions (Khatri et al., 2002).

Semi-quantitative RT-PCR

Using the software Primer3 (Whitehead Institute/MIT Center for Genome Research; http://www.genome.wi.mit.edu), sequence-specific primers were selected from the full-length cDNA sequences on the basis of published sequences of 13 invasion-associated and one housekeeping gene (Table 1). One microgram of total RNA was amplified using the One-Step RT-PCR kit (Qiagen,Valencia, CA). Reverse transcription was carried out at 55°C for 30 minutes and HotStarTaq DNA polymerase was activated at 95°C for 15 minutes followed by 25 (for all 13 invasion-associated genes) or 30 cycles (GAPDH) of amplification (94°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds), and final extension at 72°C for 10 minutes.

SDS-PAGE and western blotting

Trophoblast cell lysates were separated by SDS-PAGE and blotted as

Table 1. Differential gene expression of invasion-associated genes

GenBank Acc. No.	Gene ID	Gene name	*F/T GeneChip	*F/T RT PCR	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
U43527	KISS-1	KiSS-1	29.3	17.4	GCCATTAGAAAAGGTGGCCTC	TTGTAGTTCGGCAGGTCCTTC
AB051065	KiSS-1R	KiSS-1 receptor	n.i.	23.9	GGAGTTGCTGTAGGACATGCA	TTCGCACTGTACAACCTGCTG
L23808	MMP12	Matrix metalloproteinase 12	83.5	10.0	ACACCTGACATGAACCGTGA	AGCAGAGAGGCGAAATGTGT
Z24680	GARP	Glycoprotein A repetitions predominant	36.7	10.4	CTGCGAAACAACAGCTTCAG	GTGAGGAGGATGGCAGAGAC
M85289	HSPG2	Heparan sulfate proteoglycan 2	9.5	8.5	CTGAGTGATGCAGGCACCTA	CTCTCTGGGCTCACTTGGAC
AL050396	FLNA	Filamin A	5.0	2.0	GTCCCTGTGCATGATGTGAC	TGTATACGTGCCGTCATGGT
X66945	FGFR1	FGF receptor 1	4.3	3.2	GGGCAGTGACACCACCTACT	TGATGCTGCCGTACTCATTC
M13981	INHA	Inhibin A	3.6	2.3	GTCTCCCAAGCCATCCTTTT	CAGAGCAGAGGGAGACCAAG
M35878	IGFBP3	Insulin-like growth factor binding protein 3	2.7	1.6	ACAGCCAGCGCTACAAAGTT	AGGCTGCCCATACTTATCCA
M92287	CCND3	Cyclin D3	−2.0	−4.0	TGGATGCTGGAGGTATGTGA	GAATGAAGGCCAGGAAATCA
U15932	DUSP5	Dual specificity phosphatase 5	−2.0	−2.5	ATCAGCCAGTGTGGAAAACC	GAGACCATGCTCCTCCTCTG
U83115	AIM1	Absent in melanoma 1	−2.6	−2.0	CTGGGCCTTCTCTTTCACTG	CAACGGAAACCACTTCAGGT
AB007867	PLXNB1	Plexin B1	−14.7	−3.8	ACAGGCAAGGCCAAATACAC	CTCATCACTTGGCTTCACCA
X01677	GAPDH	Glyceraldehyde-3phosphatedehydrogenase	1.2	1.0	TGAAGGTCGGAGTCAACGGAT	GTCATGAGTCCTTCCACGATA

*F/T, Fold change difference of mRNA levels between FT vs term trophoblasts; n.i., not included on the U95A GeneChip.

described previously (Hahn et al., 1998). Blots were incubated with Kp-54 antiserum (1:10,000). To determine the apparent molecular weight of the band identified by the antiserum, a standard curve was generated by plotting the log10 of unstained molecular weight standards against their retention factor (Rf). Thus, a single band at ~15 kDa could be identified as Kp-145.

MALDI-TOF analysis

Conditioned media from FT trophoblasts cultured for 48 hours in serum-free DMEM were precleared from excess protein over a Nucleosil RP-18 column (Macherey Nagel, Easton, PA). The peptides were eluted with a linear gradient of acetonitrile (10-90%) in 0.1% (v/v) trifluoroacetic acid (TFA) over 30 minutes with a flow rate of 0.4 ml/minute; fractions were collected from 10.7 to 13 minutes. The synthetic peptides (Kp-10, -13, -14 and -54) were analyzed in a separate run to determine their retention times. In a second purification step, purified fractions were reconstituted in 10% (v/v) acetonitril containing 0.1% (v/v) TFA and passed over a Vydac RP-18 column (Vydac, USA). Peptides were eluted over 15 minutes with the same linear gradient as above. Fractions containing Kisspeptins were collected according to the retention times of the synthetic Kisspeptins and subjected to MS-Analysis. MALDI-TOF spectra were obtained in positive linear mode on a KRATOS Axima CFR mass spectrometer (Manchester, UK) by integrating 20 to 50 laser shots per spectrum. Alpha-Cyano-4-hydroxycinnamate saturated in 30% (v/v) acetonitrile and 0.1% (v/v) TFA in water was used as a matrix. External calibration was performed with adrenocorticotropic hormone (ACTH) fragment 18-39; 2466.71 m/z [MH+ av.], and insulin 5734.6 m/z [MH+ av.] (used as the mass calibration standards).

Immunohistochemistry

Deparaffinized tissue sections were treated for 10 minutes with blocking solution, then incubated for 30 minutes with primary antibodies (rabbit polyclonal antiserum against Kp-54, 1:500; anti-KiSS-1R, 1:100) followed by an incubation with an appropriate secondary antibody for 15 minutes, and then 15 minutes with avidin-biotin-peroxidase (Labvision). Immunolabeling was visualized by a 5 minute exposure to 3-amino-9-ethylcarbazole. Sections were then counterstained for 3 minutes with hematoxilin and mounted in Faramount (DAKO).

In situ hybridization

FT or term placenta sections were deparaffinized and hybridized with digoxygenin (dig)-labeled riboprobes to KiSS-1 and KiSS-1R, according to a published protocol (Simeone, 1998). RNA probes for human KiSS-1 and KiSS-1R were prepared as follows: on the basis of the published sequence, a 457 bp fragment of the the human KiSS-1 cDNA sequence was amplified by PCR using a primer pair KiSS-1_*Eco*RI (5′-GGG AAT TCT AGA CCC ACA GGC CAG CAG CTA GAA-3′; bp 296-328) and KiSS-1_*Hin*dIII (5′-TTT ATT GCC TAA GCT TGG AAG CTC CAG CGC CCC-3′; bp 724-752). Similarly, a 781 bp fragment of the human KiSS-1R cDNA sequence was amplified by PCR using primer sets KiSS-1R_*Eco*RI (5′-TGG GGA ATT CGC TGG TCA TCT ACG TCA TCT G-3′;) and hOT75T175_*Hin*dIII (5′-CAG GTC TTA AGC TTG TAG GCG GCG TAG CTG-3′). The amplified sequences were confirmed at The Scripps Research Institute's DNA Sequencing core facility (http://www.scripps.edu/). Amplified fragments were subcloned into the *Eco*RI and *Hin*dIII sites of pGEM-3Z (Promega, Madison, WI), and the resultant vectors were used as templates for construction of the dig-labeled RNA probes according to manufacturer's instructions (Roche Diagnostic, Indianapolis, IN). After hybridization, the slides were washed, and incubated for 4 hours at RT with peroxidase-labeled goat-anti dig monoclonal antibody. Signals were detected overnight at RT in buffer 2 containing 3 μl/ml NBT (Nitroblue tetrazoliuim chloride), 50 mg/ml BZIP (5-bromo-4-chloro-3-indolyl-phosphate, 4-

toluidine salt) and 1 mM Levamisol. Color development was terminated in TE (10 mM Tris/HCl; pH 8.0, 1 mM EDTA) for 5 minutes. Sections were mounted in gelatin-glycerol (Sigma).

Explant culture

The explant culture method followed a standard protocol (Aplin et al., 2000). Dissected mesenchymal villous tissue was arranged radially on 80 µl drops of collagen type I gel, covered with 20 µl of serum-free medium (SFM; DMEM:Ham's F12 (Gibco) 1:1, supplemented with 100 µg/ml streptomycin/penicillin (Gibco) and incubated overnight at 37°C, 5% CO_2 to allow attachment. Wells were then carefully flooded with 1 ml SFM and incubated in the absence or presence of 0.3 or 1.0 µM Kp-10 at 37°C, 5% CO_2 and subsequent growth characteristics monitored by light microscopy. Newly synthesized DNA was detected with bromodeoxyuridine (BrdU; Roche) added to selected cultures at 1 µM for the periods specified. The extent of migration, i.e. the distance from the cell column base to the tip of the outgrowth, was measured at three defined positions (three explants per condition) and the mean of these values was used for comparing the Kp-10 effects.

Intracellular Ca^{2+} release assay

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) in single cells was measured using the conventional single-cell fura-2 technique with a customized fluorescence microscope as described previously (Graier et al., 1998), and expressed as ratio (F_{340}/F_{380}) units. Briefly, 1×10^5 FT trophoblasts were plated on glass coverslips. The following day, adherent cells were loaded for 45 minutes at RT in the dark with 2 µM fura-2/AM (Molecular Probes, Leiden, The Netherlands), washed twice and equilibrated for a further 20 minutes. The coverslip was mounted into an experimental chamber and perfused (1 ml/min^{-1}) with buffer solution containing, in mM: 145 NaCl, 5 KCl, 2 Ca_2Cl, 1 $MgCl_2$ and 10 Hepes free acid (pH adjusted at 7.4).

Migration assays

Transwell migration assays were performed as described (Hintermann et al., 2001; Song et al., 2001) with filters coated with 30 µg/ml collagen I or 10 µg/ml fibronectin. After initial experiments to determine the temporal kinetics of migration, subsequent migration assays were performed for 48 hours. Cells that had migrated through the filters were viewed under bright-field optics and were counted in eight fields (using a 20× objective) from each of two filters for each condition, determining the mean number of cells counted per field.

Gelatin zymography

Protein content of trophoblastic conditioned media was measured according to the method of Bradford (Bradford, 1976), and equal amounts of protein were subjected to gelatin zymography on nonreducing 10% polyacrylamide gels supplemented with 1% gelatin to determine the proteolytic activity of conditioned media as described previously (Xu et al., 2001). Prestained molecular mass standards (Invitrogen) were used to determine the molecular mass of proteolytic activity.

Statistical analysis

Migration indices and MMP-2 levels were analyzed using one-way ANOVA, followed by the Tukey test. As data were not normally distributed, the Mann-Whitney rank-sum test was employed to determine the level of significance of differences in pairs of various treatment groups. $P<0.05$ was considered significant.

Results

Screening for invasion-related genes in human trophoblasts, using DNA microarray technology

DNA microarray-based large-scale gene expression profiling was applied to highly (FT) and poorly (term of gestation) invasive trophoblast cells. In all, 643 differentially expressed genes (more than twofold difference between FT and term trophoblast cells) were further compared with the gene expression profile of the poorly invasive but proliferative trophoblast-like cell line BeWo (Grummer et al., 1994; Morgan et al., 1998; Xu et al., 2001). Genes that showed a similar

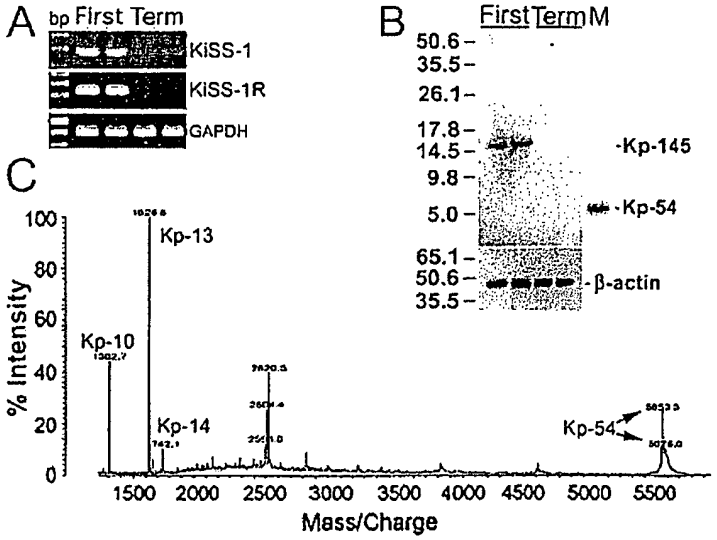

Fig. 2. KiSS-1 and KiSS-1R are differentially expressed in FT and term human trophoblast cells. (A) RT-PCR of total RNA of freshly isolated FT or term trophoblasts (representative experiment: left panel). (B) Western blotting of FT and term trophoblasts. Lysates were probed with anti-Kp145/Kp-54 antiserum. With purified Kp-54 (5 ng) the antiserum produced a single band at 5-6 kDa, which is in good agreement with the theoretical MW for Kp-54 (5858.0 kDa; lane 'M'). The antibody reacted only with lysates from FT trophoblasts producing one prominent band at ~15-16 kDa, which is in good agreement with the calculated M_r for Kp-145 (15.391 kDa). β-actin served as loading control. (C) FT trophoblast conditioned media were subjected to MALDI-TOF analysis after reverse phase-HPLC fractionation. The theoretical masses for C-terminally amidated Kps-10, -13, -14 and -54 are 1304, 1626, 1704 and 5858, respectively. Kp-54 was identified also as Na-adduct (m/z: 5876). The masses at around 2600 are unidentified.

expression pattern in both FT and BeWo cells were assumed to control processes common to both cell types, including proliferation and endocrine function, but not invasion. Thus, BeWo cells served as a 'filter' to identify genes regulating trophoblast invasion. Using this selection method, 220 invasion-associated genes were differentially expressed, some of which are shown in Table 1. A similar approach was used to identify prognostic biomarkers in prostate cancer (Dhanasekaran et al., 2001). In that study, distinct noninvasive prostate samples clustered together, differently from the metastatic samples. In order to identify novel key players regulating trophoblast invasion we focused on KiSS-1, a recently described tumor suppressor gene (Ohtaki et al., 2001), as it was one of the strongest differentially expressed genes (29.0-fold higher mRNA levels in FT compared with term trophoblasts).

Semi-quantitative RT-PCR and western blot analysis

To confirm differential expression of KiSS-1 mRNA and to include KiSS-1R in our studies, we performed semi-quantitative RT-PCR and found 17.4±3.4-fold (KiSS-1) and 23.9±5.4-fold (KiSS-1R) higher mRNA levels in FT than term trophoblasts (Fig. 2A). In western blot analyses, the antiserum generated against Kp-54, which is within the Kp-145 sequence, detected Kp-145, but not Kp-54, in lysates from FT trophoblasts (Fig. 2B) but not in FT trophoblast conditioned medium, even after 30-fold enrichment (data not shown). This suggests the following

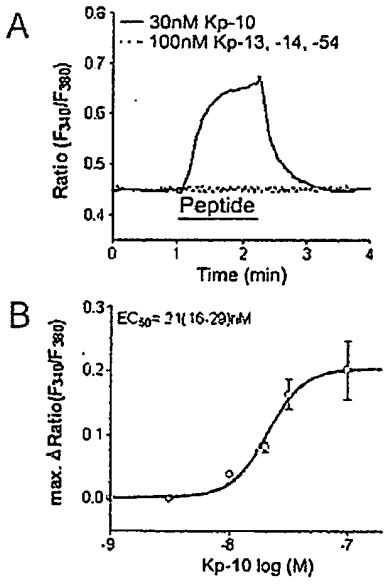

Fig. 3. Kisspeptin-10 raises intracellular Ca^{2+} in isolated first trimester human trophoblasts. FT trophoblasts were stimulated with different Kps and intracellular [Ca^{2+}] was measured (A). Only Kp-10 resulted in an increase in intracellular [Ca^{2+}] (n=9), whereas Kp-13, -14 and -54 were ineffective (n=6). (B) Concentration-response curve for Kp-10 on intracellular free Ca^{2+} concentration in isolated FT trophoblasts. The intracellular [Ca^{2+}] is expressed as ratio (F_{340}/F_{380}) (mean±s.e.m.; n=6-9). The EC_{50} was found to be 21 (16-29) nM (95% confidential interval).

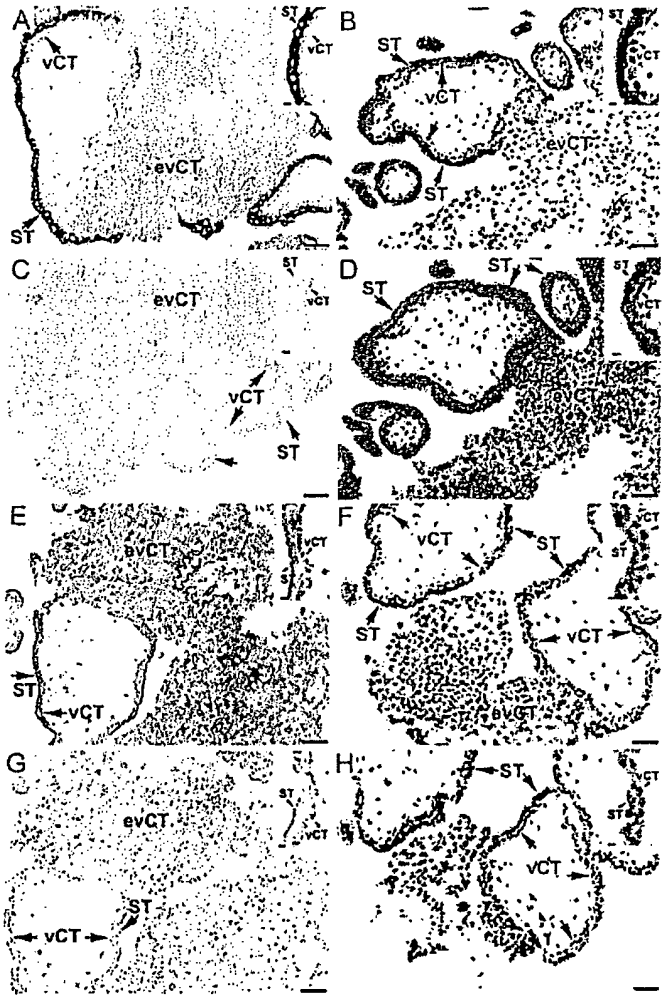

Fig. 4. KiSS-1/Kp-54 and KiSS-1R localization in first trimester placenta. Sections of human FT placental tissue at week 6-10 of gestation showing the localization of KiSS-1/Kp-54 (A,B) and KiSS-1R (E,F). KiSS-1 and KiSS-1R mRNA were detected by in situ hybridization as dark blue precipitates, whereas their respective proteins were detected using affinity purified polyclonal antibodies evident as dark red precipitate. Sense probes (C,G) and nonimmune sera (D,H) produced no detectable signal. *KiSS-1* mRNA (A) and Kp-145/Kp-54 protein (B) were detected mainly on the outer (syncytiotrophoblast) surface of villi. Higher magnification showed that *KiSS-1*/Kp-54 expression is restricted to the syncytiotrophoblast (insets; A and B; bars, 25 μm), whereas it was undetectable in villous (vCT) and extravillous (evCT) cytotrophoblasts. *KiSS-1R* mRNA is located in the syncytiotrophoblast (ST), villous (vCT) and extravillous (evCT) cytotrophoblasts (E). This mRNA staining pattern is paralleled by that of KiSS-1R protein (F). Bars, 50 μm.

scenarios: (1) intracellular Kp-54 pools are rapidly depleted by secretion; (2) intracellularly generated Kp-54 is rapidly degraded because of the presence of PEST sequences (rich in proline, glutamic acid, serine, threonine and aspartic acid residues predisposing proteins to ubiquitination and proteosome degradation (Harms et al., 2003). The presence of this motif (aa 68-87) may allow rapid degradation of cytosolic Kp-145 resulting in a short half-life (<30 seconds) (Harms et al., 2003); (3) levels of intra- or extracellular Kp-54 are below the detection limit of western blotting. To exclude the latter, conditioned medium of cultured FT trophoblasts was analyzed by MALDI-TOF, which revealed the presence of Kp-10, -13 and -14, as well as Kp-54 in conditioned medium, with masses consistent with the theoretical masses of the amidated forms for Kp-10, -13, -14 and Kp-54 (Fig. 2C). Secreted Kp-54 may be the source for the shorter Kps Kp-10, -13 and -14.

Next, we tested which of the various Kps can activate KiSS-1R by monitoring cytosolic Ca^{2+} levels after stimulation of isolated FT trophoblasts with the respective Kps (Fig. 3). This treatment increased intracellular Ca^{2+} in KiSS-1R-overexpressing cells (Ohtaki et al., 2001; Muir et al., 2001; Kotani et al., 2001). Addition of Kp-10 to FT trophoblasts increased cytosolic Ca^{2+} levels with an EC_{50} of 21 nM (Fig. 3A,B). By contrast, Kp-54 as well as Kp-13 and Kp-14 failed to produce this response (Fig. 3A).

Localization of KiSS-1 and KiSS-1R mRNA and protein in first trimester placenta

Both KiSS-1 mRNA and protein were found at the syncytiotrophoblast of anchoring (Fig. 4A,B) and floating villi, whereas it was undetectable in villous and extravillous cytotrophoblasts. Maternal decidua was devoid of KiSS-1 mRNA staining (data not shown). On the basis of these results, expression of KiSS-1 as well as KiSS-1 encoded Kps in FT placenta appears to correlate with cells that have fused and become noninvasive. KiSS-1R mRNA and protein was localized to syncytio-, villous- and extravillous cytotrophoblast at similar levels (Fig. 4C,D and insets). Thus, syncytiotrophoblast expresses both the ligand Kp-145/Kp-54 and its receptor, whereas the extravillous cytotrophoblast only expresses the

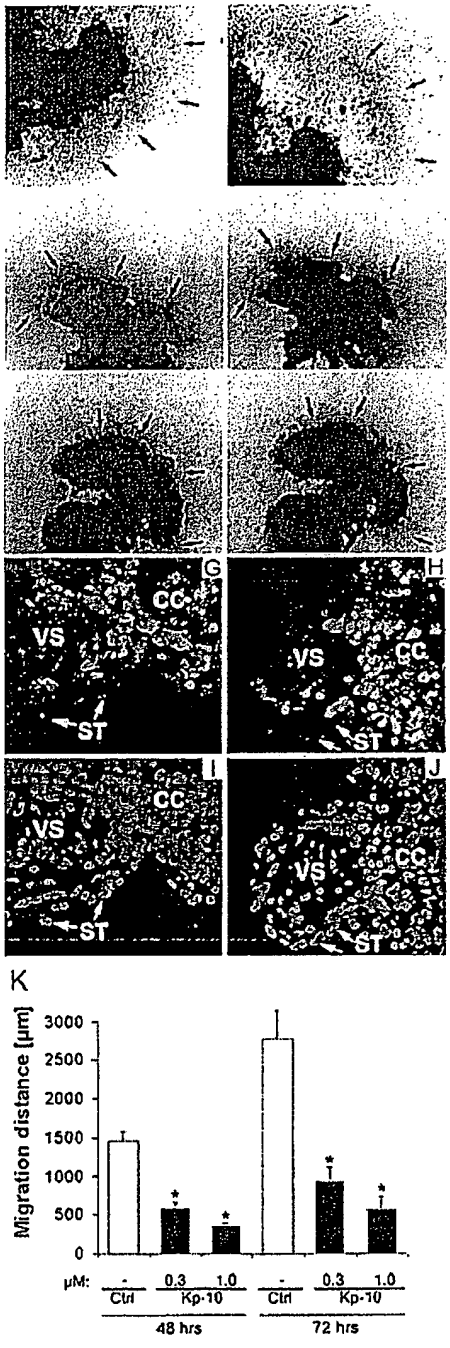

Fig. 5. Kisspeptin-10 inhibits trophoblast outgrowth and migration, but not proliferation in first trimester human villous explant cultures. Villous explants from 6-9 weeks' gestation were maintained in culture for 72 hours in the absence (A,B) or presence of 0.3 µM (C,D) or 1.0 µM (E,F) Kp-10. Identical villi were photographed at 48 or 72 hours. The dark areas are tissue, and sheets of outgrowing cytotrophoblast can readily be observed in the untreated cultures (A,B; arrows mark the limits/boundaries of the outgrowth). Kp-10 treatment profoundly decreases trophoblast outgrowth from the distal end of the villous tips when compared with control villous explants. Magnification: ×40. (G-J) Proliferative potential of placental villi was determined by incorporation of BrdU after 24 hours in culture in the absence (G,I) or presence (H,J) of 1 µM Kp-10. Villus sections were stained with an anti-BrdU antibody, which detects cells in the S-phase (G,H), or were labeled with the nuclear stain dapi (I,J). The nonproliferating syncytiotrophoblast (ST) is devoid of anti-BrdU staining (G,H). Cell column (CC) formation by villus explants maintained in the absence or presence of Kp-10 (0.3 µM) was similar. In addition, no significant difference in the proportion of nuclei that incorporated BrdU was detected. Six villi per treatment were examined per placenta and the experiment was repeated three times. Magnification: ×400. The extent of migration was increased between 48 and 72 hours under all conditions. Migration distance was reduced already at 0.3 µM Kp-10 at both 48 and 72 hours (K). The higher concentration of 1 µM did not augment the effect. *P<0.05 vs control. VS, villous stroma.

receptor KiSS-1R (Fig. 7). This is in line with the hypothesized inhibitory role of Kp-54 in trophoblast invasion.

Regulation of trophoblast migration by Kp-10 in vitro

Given the anti-metastatic function of KiSS-1, we investigated whether Kp-10 can inhibit migration of FT placental tissue. Tissue cultures of anchoring villi explanted from early gestation (6 to 9 weeks) placentas onto an extracellular matrix substrate allow the study of trophoblast outgrowth, migration and invasion, thus mimicking the key processes that occur in anchoring villi during the FT of gestation (Genbacev et al., 1992; Aplin et al., 2000). This assay has the significant advantage of allowing migratory behaviour to be observed in living trophoblasts, because migration occurs largely across the surface of the gel. Control explants displayed prominent outgrowth of extravillous trophoblast from the distal end of the villous tip and an increased number of cells migrating into the surrounding matrix (Fig. 5A,B) similar to previous reports (Aplin et al., 2000). Kp-10 strongly affected the pattern of cell migration (ANOVA $P<0.001$): explants exposed to Kp-10 for 48 or 72 hours displayed about 70% diminished extravillous trophoblast outgrowth (Fig. 5C-F,K).

Because de novo cell column formation is accompanied by cytotrophoblast proliferation peaking after an initial period of 24 hours and ceasing after 48 hours (Caniggia et al., 1997; Genbacev et al., 1992; Aplin et al., 2000), we investigated whether the Kp-10-induced inhibition of trophoblast outgrowth was due to a reduction in cell division. When tissue was explanted in the presence of Kp-10, viability and proliferative potential of cytotrophoblast was maintained for the first 24 hours as indicated by the incorporation of BrdU into cytotrophoblasts beneath the villous syncytium and in the proximal cell column (Fig. 5). Because cytotrophoblast proliferation was unaffected in the critical phase, these results indicate that Kp-10 does not affect trophoblast proliferation and that the reduced trophoblast outgrowth is most likely due to a blockade of trophoblast migration. This was tested in a Transwell migration assay, where inhibition of FT trophoblast migration was readily apparent by visual inspection (Fig. 6A). The number of migrating cells scored per field was reduced by 37% and 46% in the presence of 0.5 μM Kp-10 on fibronectin or collagen I, respectively (Fig. 6B). Like in the explant assay, Kp-10 did not alter FT trophoblast proliferation (Fig. 6B).

Regulation of cell motility is controlled by a multitude of factors (Lauffenburger and Horwitz, 1996), among which proteases play an evident role (Bischof and Campana, 2000; Chang and Werb, 2001). To determine whether Kp-10 triggers a decrease in collagenase activity, resulting in reduced trophoblast migration, FT trophoblast-conditioned medium was analyzed for collagenase activity by zymography. Addition of 0.3 μM or 1.0 μM Kp-10 to FT trophoblast cultures suppressed MMP-2 activity by 32% and 62% after 24 hours, and by 28% and 42% after 48 hours, respectively (Fig. 6C,D). Secreted MMP-9 levels were too low to allow accurate quantification (data not shown).

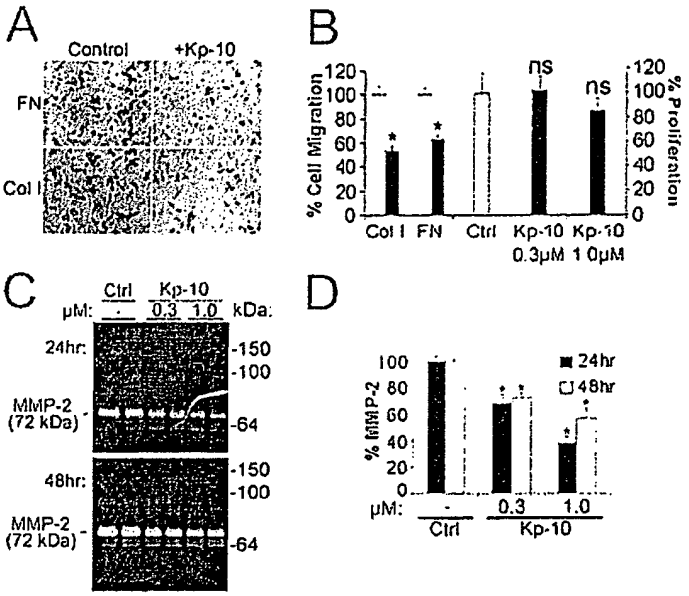

Fig. 6. Kisspeptin-10 inhibits migration and gelatinolytic activity, but not proliferation of isolated first trimester human trophoblasts. (A) Microscopic image of the abluminal side of collagen I (Col I)- or fibronectin (FN)-coated membranes after 48 hours during which isolated FT trophoblasts (1×10^5 per filter, week 6-9 of gestation) migrated across the membrane in the absence or presence of 0.5 μM Kp-10. (B) Bars on left: Addition of Kp-10 (0.5 μM, black bars) reduced the number of cells that migrated through the filter pores. Results are expressed as mean±s.e.m. (n=3 different trophoblast isolations) of cell migration relative to unstimulated cells set as 1 (white bars) (*$P<0.05$ vs control). Bars on right: To assess cell proliferation, 7×10^4 freshly isolated FT trophoblasts were plated on collagen I in the absence (white bar) or presence of Kp-10 (black bars) added at the time of seeding. Cell numbers were counted after 48 hours. Results are presented as cell number (mean±s.e.m.; n=3) expressed relative to control (=100%). Kp-10 did not affect FT trophoblast proliferation significantly. ns, not significant vs control. (C) Conditioned medium from isolated FT trophoblasts plated on collagen I for 24 or 48 hours was subjected to gelatin zymography. Proteolytic activity was noted for the 72-kDa collagenase corresponding to pro-MMP-2. Protein molecular weight markers are indicated on the right. (D) Addition of Kp-10 suppressed 72-kDa collagenase (MMP-2) activity (*$P<0.01$ vs control).

Discussion

In the present paper we show that kisspeptin Kp-10, a shorter version of the recently described metastasis suppressor gene product KiSS-1, inhibits trophoblast migration and invasion, presumably by downregulating protease activity. Our hypothesis is based on the finding that KiSS-1 was among the strongest differentially expressed genes in invasive FT versus noninvasive term trophoblast preparations. Furthermore, Kp-10 blocked explant invasion and Transwell migration and

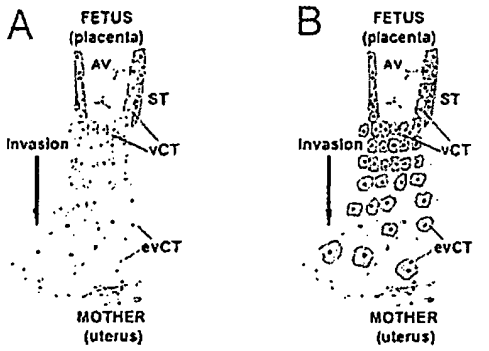

Fig. 7. KiSS-1 and KiSS-1R mRNA and protein expression in first trimester placenta. The histology of the maternal-fetal interface and KiSS-1 (A) and KiSS-1R (B) expression patterns are shown schematically from in situ hybridization and immunohistochemistry staining (Fig. 4). AV, anchoring villus; evCT: extravillous cytotrophoblast; ST, syncytiotrophoblast; vCT, villous cytotrophoblast.

decreased MMP-2 activity. These results make KiSS-1 a strong candidate as a gene that regulates trophoblast invasion in vivo.

Differential expression of KiSS-1 was similar in microarray and RT-PCR experiments. These results confirm published data on the relative expression levels of the well-known invasion-specific trophoblast markers integrins $\alpha 1$ and $\alpha 5$, as well as HLA-G, which are almost exclusively expressed by the invasive cytotrophoblast phenotype (Damsky et al., 1994; McMaster et al., 1998; Cervar et al., 1996; Blaschitz et al., 2000), thus confirming our microarray strategy. Differential expression of KiSS-1 was paralleled by the expression and location of its receptor KiSS-1R, suggesting autocrine interactions. Additional receptor expression on villous and extravillous cytotrophoblast also suggests paracrine or endocrine mechanisms for receptor activation (Fig. 7, Fig. 3). This is supported by the findings that Kp-10 stimulates oxytocin secretion in rats and that pregnant women have high Kp-54 plasma levels (Horikoshi et al., 2003; Kotani et al., 2001). However, the cellular origin of secreted Kp-54 has not been shown so far. Here, Kp-54 and its fragments Kp-10, -13 and -14 were identified by MALDI-TOF analysis in serum-free conditioned medium of isolated FT trophoblasts. Kp-54 is predicted to result from the proteolytic processing of Kp-145 by furin or prohormone convertases, as its sequence is surrounded by pairs of basic residues in the full-size protein (Harms et al., 2003). At present, the mechanism resulting in the generation of Kp-10, -13 and -14 is unclear as no obvious cleavage sites have been found (Harms et al., 2003), but scenarios such as spontaneous decomposition as well as constitutive or regulated processing can be envisioned.

In cells overexpressing KiSS-1R, Kp-54 was capable of activating the receptor and increasing intracellular Ca^{2+} levels (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). In contrast to the present system with a physiological receptor level, only Kp-10 gave rise to increasing intracellular Ca^{2+} with an EC_{50} similar to other systems (Kotani et al., 2001; Muir et al., 2001). Because the Kp-54 used here caused a rapid Ca^{2+} response in KiSS-1R-overexpressing cells (Muir et al., 2001) we conclude that the chemically synthesized Kp-54 can induce effects in cells overexpressing KiSS-1R, whereas Kp-10 is the activator in physiological systems. The special position of Kp-10 is highlighted not only because of its threefold-to-tenfold higher receptor affinity than other Kps (Hori et al., 2001; Ohtaki et al., 2001), but also by the high conservation of the Kp-10 sequence between mouse and human with one conserved amino acid replacement, whereas other regions of the Kp-145 protein display only low homology (Stafford et al., 2002).

The extent of trophoblast invasion also depends on the provision of trophoblasts from the cell column that will differentiate and acquire an invasive phenotype. Explant and Transwell migration assays showed that Kp-10 signals reduced trophoblast motility without affecting trophoblast proliferation or viability. The absence of Kp-10 effect on trophoblast proliferation shows that the mechanism underlying invasion inhibition must be different. In fact, suppressed motility was associated with suppressed MMP-2 proteolytic activity. The high in vitro invasive capacity of FT trophoblasts has been shown to depend on their secretion of collagenases and gelatinases (Bischof et al., 1995; Bischof and Campana, 2000). Indeed, extravillous cytotrophoblasts secreting high amounts of gelatinases are highly motile and invasive, whereas extravillous cytotrophoblasts secreting low amounts of gelatinases have become immobile. Thus, syncytium-derived Kp-10, by suppressing gelatinolytic activity of extravillous cytotrophoblast located in proximity to maternal circulation, might be an endocrine mechanism of fine-tuning trophoblast motility (Fig. 7). In addition, syncytial Kp-10 may also act in a paracrine manner on the underlying villous cytotrophoblast by suppressing gelatinolytic activity in a coordinated fashion with other signals, thus rendering villous cytotrophoblasts immobile and anchored to the villous basement.

Although the ability of Kp-10 to inhibit migration has been documented in a few other cell types with overexpression of KiSS-1R (Ohtaki et al., 2001), this is the first study to report Kp-10 motility regulation in a human physiological setting. Our conclusion of an invasion inhibiting function of the KiSS-1/KiSS-1R system in human trophoblasts is corroborated by reduced expression of both in trophoblast-derived choriocarcinoma (Janneau et al., 2003). Invasion inhibition will lead to excessive invasion that is a characteristic of this tumor.

Downregulation of MMP-2 activity may be one potential mechanism underlying the Kp-10-induced invasion inhibition and this may be tissue specific (Yan et al., 2001). The possibility that Kp-10 affects MMP-9 expression or activity later in gestation cannot be excluded because trophoblast-derived MMPs are developmentally regulated throughout pregnancy and MMP-9 secretion increases between week 6 and 11 of gestation (Xu et al., 2001; Huppertz et al., 1998). In addition to MMP regulation, probably by direct interaction with MMPs (Takino et al., 2003), other mechanisms may be operative, such as activation of focal adhesion kinase (Kotani et al., 2001; Ohtaki et al., 2001; Ilic et al., 2001), alteration of tissue inhibitors of MMPs (TIMPs) or effects on the plaminogen activator/inhibitor system. The absence or low levels of invasion in situ at term despite low KiSS-1 levels suggests that other anti-invasive factors prevail at the end of gestation. Hence, the anti-invasive effect of KiSS-1/KiSS-1R system may only be important in the first trimester.

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Continuous Human Metastin 45–54 Infusion Desensitizes G Protein-Coupled Receptor 54-Induced Gonadotropin-Releasing Hormone Release Monitored Indirectly in the Juvenile Male Rhesus Monkey (*Macaca mulatta*): A Finding with Therapeutic Implications

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The effect of continuous administration of the C-terminal fragment of metastin, the ligand for the G protein-coupled receptor, GPR54, on GnRH-induced LH secretion was examined in three agonadal, juvenile male monkeys whose responsiveness to GnRH was heightened by pretreatment with a chronic pulsatile iv infusion of synthetic GnRH. After bolus injection of 10 μg human (hu) metastin 45–54 (equivalent to kisspeptin 112–121), the GPR54 agonist was infused continuously at a dose of 100 μg/h and elicited a brisk LH response for approximately 3 h. This rise was then followed by a precipitous drop in LH despite continuous exposure of GPR54 to metastin 45–54. On d 4, during the final 3 h of the infusion, single boluses of hu metastin 45–54 (10 μg), *N*-methyl-DL-aspartic acid (NMDA) (10 mg/kg) and GnRH (0.3 μg) were administered to interrogate each element of the metastin-GPR54-GnRH-GnRH receptor cascade. Although the NMDA and GnRH boluses were able to elicit LH pulses, that of hu metastin 45–54 was not, demonstrating functional integrity of GnRH neurons (NMDA) and GnRH receptors (NMDA and GnRH) but desensitization of GPR54. The desensitization of GPR54 by continuous hu metastin 45–54 administration has therapeutic implications for a variety of conditions currently being treated by GnRH and its analogs, including restoration of fertility in patients with abnormal GnRH secretion (*i.e.* idiopathic hypogonadotropic hypogonadism and hypothalamic amenorrhea) and selective, reversible suppression of the pituitary-gonadal axis to achieve suppression of gonadal steroids (*i.e.* precocious puberty, endometriosis, uterine fibroids, and prostate cancer). (*Endocrinology* 147: 2122–2126, 2006)

THE TRIGGERS FOR the resurgence of GnRH secretion at the time of puberty in primates are as mysterious as those that halt its secretion at the end of the infantile period. Several approaches have been employed to identify these elusive signals, including genetic studies using DNA from patients with reproductive disorders. Loss-of-function mutations in the gene encoding the G protein-coupled receptor, GPR54, have recently been demonstrated to cause hypogonadotropic hypogonadism, a condition characterized by an absence of pubertal development (1, 2). Across species, *Gpr54* knockout mice are phenocopies of this syndrome (2).

The endogenous ligand of GPR54 is derived from kisspeptin-1, which is proteolytically processed in man to a 54-amino-acid peptide, human (hu) metastin (3–5). Metastin's name was so coined because of its ability to suppress metastases of human melanomas and breast carcinomas (6, 7). When administered as a single bolus to mice (8), rats (9–13), and agonadal, juvenile, male monkeys (14), hu metastin and hu metastin 45–54 (or the mouse analog) elicit a robust LH response that is blocked by previous treatment with a GnRH receptor antagonist and, therefore, presumably mediated through GnRH release from the hypothalamus (8, 14). When administered as an iv infusion to ovariectomized, estradiol-treated sheep (4 h), hu metastin 45–54 also stimulates LH release and GnRH levels are increased in cerebrospinal fluid (15). In human males, an iv infusion (90 min) of hu metastin also stimulates LH release (16). Outside of these single boluses and brief infusions, chronic, intermittent administration of hu metastin 45–54 (or the mouse analog) induces early sexual maturation in immature female rats (13) and sustained and precocious GnRH release in juvenile male monkeys (17). However, the effect of long-term continuous metastin administration on the hypophysiotropic drive to the gonadotrophs is unknown. The goal of this study was to test the hypothesis that administration of a 4-d, continuous infusion of high-dose hu metastin 45–54 to juvenile, agonadal male rhesus monkeys stimulates sustained gonadotropin release and, by extension, sex steroid secretion.

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Abbreviations: DMSO, Dimethylsulfoxide; DPBS, Dulbecco's PBS; GPR, G protein-coupled receptor; hu, human; NMDA, *N*-methyl-DL-aspartic acid.

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Materials and Methods

Animals

Three juvenile male rhesus monkeys (*Macaca mulatta*, 19–20 months of age, 2.6–3.8 kg body weight) were used. The age of the animals at the

end of the study was 21–23 months, and the pubertal reactivation of the hypothalamic-pituitary axis under the conditions of the present experiment usually occurs from 24–30 months of age (18). The animals were maintained under controlled photoperiod (lights on from 0700–1900 h) and at approximately 21 C in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Reagents

Metastin, the ligand for GPR54, is proteolytically processed from its parent protein, kisspeptin-1. This paper employs the term metastin with amino acid numbering reflecting this protein/peptide specifically (C-terminal fragment of hu metastin 45–54 is equivalent to hu kisspeptin-1 112–121). The C-terminal fragment of hu metastin (hu metastin 45–54) was selected for these studies because it has been shown to have comparable biological potency as the full-length peptide (4). The hu metastin 45–54 dose chosen for continuous administration (100 μg/h iv) was based on previous studies (14) in which bolus iv injections of 100 μg of this peptide were found to stimulate GnRH-induced LH secretion in agonadal, juvenile monkeys. Hu metastin 45–54 was synthesized at the Peptide/Protein Core Facility of the Massachusetts General Hospital. A stock solution of the peptide (500 μg/ml) was prepared in 5% dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO) in physiological saline (0.9% NaCl) (Abbott Laboratories, Chicago, IL) and stored at −80 C. For continuous administration, the hu metastin 45–54 infusate (100 μg/3 ml) was prepared the day before the experiment began by diluting the stock preparation with sterile Dulbecco's PBS (DPBS) without $CaCl_2$ and $MgSO_4$ (Life Technologies, Inc. Products, Grand Island, NY) and stored at 4 C. During the experiment, a calibrated reservoir (Buretrol; Baxter Healthcare Corp., Deerfield, IL) containing the infusate was maintained at room temperature and refilled every 24 h. A stock 5% DMSO solution in sterile saline was prepared and stored at −80 C. For vehicle infusion, this was diluted 1:15 with sterile DPBS, stored at 4 C, and used as described for the hu metastin 45–54 infusate.

For bolus administration of hu metastin 45–54, a 10-μg dose (10 μg/ml in sterile DPBS) was used. This bolus dose was chosen, in part, because of the recent demonstration that repetitive 2-μg boluses of hu metastin 45–54 administered once every hour are capable of inducing LH discharges in agonadal, juvenile male monkeys (17). However, because a 2-μg bolus of hu metastin 45–54 infused over 1 min is roughly equivalent to the continuous infusion rate (100 μg/h = 1.7 μg/min), a higher dose of 10 μg was chosen for the bolus challenges in this experiment. *N*-Methyl-DL-aspartic acid (NMDA) (Sigma-Aldrich Inc., St. Louis, MO) was dissolved in sterile saline at a stock concentration of 50 mg/ml. On the days of NMDA administration, doses of 10 mg/kg body weight were prepared in 1 ml sterile saline and passed through a 0.22-μm filter (Fisher Scientific, Pittsburgh, PA) before injection.

GnRH, synthesized at the Salk Institute (Contract N01-HD-0-2906), was obtained from the National Hormone and Peptide Program. A stock GnRH solution was prepared at 1 mg/ml in sterile saline and stored at −20 C. For intermittent infusion, GnRH was diluted to 0.3 μg/ml in saline, stored at −20 C, and used as required.

Surgical procedures

Bilateral castration and implantation of iv catheters (inner diameter, 0.040 in; outer diameter, 0.085 in) (Stuart Bio-Sil; Sil-Med Corp., Taunton, MA) were performed under sterile conditions as described previously (18). Briefly, the animals were sedated with ketamine hydrochloride (10–20 mg/kg body weight, im) (Ketaject; Phoenix Scientific Inc., St. Joseph, MO) and anesthetized by isoflurane inhalation (1–2%, in oxygen) (Abbott Animal House, North Chicago, IL). Bilateral castration was performed a few weeks before or at the time of catheterization. Two indwelling catheters were employed, one placed in an internal jugular or subclavian vein and the other in a femoral vein. During the continuous infusion of hu metastin 45–54, one line was dedicated to infusion and one to sampling. The animals received a single im injection of penicillin (Pen-G, 40,000 U/kg body weight) (Phoenix Scientific) on the day of surgery. Postsurgically, the animals received twice-daily iv injections of a broad-spectrum antibiotic (Kefzol, 25 mg/kg body weight) (Apothecon, Princeton, NJ) and an analgesic (Ketofen, 2 mg/kg body weight) (Fort Dodge Animal Health, Fort Dodge, IA) for 4 d. The routine maintenance of animals in remote sampling cages has been described previously (18).

Collection of blood samples

Blood samples (1 ml) were withdrawn via the sampling catheter into heparinized syringes and transferred to sterile tubes, and the plasma was harvested after centrifugation. During periods of sequential sampling, packed blood cells were resuspended with sterile saline and returned to the respective animal. Plasma was stored at −20 C until required for assays.

In situ GnRH bioassay

To use pituitary LH secretion as a bioassay for endogenous GnRH release in juvenile animals, the responsiveness of the gonadotrophs to GnRH stimulation was first enhanced by a chronic pulsatile iv infusion of GnRH (0.15 μg/min for 2 min every hour), as described on several occasions previously (14, 18–21). A robust, adult-like LH response to exogenous GnRH stimulation is usually established by approximately 3–4 wk of pulsatile GnRH treatment (21). After termination of the priming infusion, circulating LH concentrations fall rapidly to undetectable levels, but the response of the pituitary to GnRH is maintained for several days (18), allowing experimentally induced endogenous GnRH release to be easily detected. GnRH priming was reestablished between the hu metastin 45–54 and vehicle infusions.

Experimental design

The experiment was initiated after 4–5 wk of intermittent priming with GnRH and after confirmation that pituitary responsiveness to GnRH had been markedly up-regulated by this treatment. At this time (d 1), the iv intermittent infusion of GnRH was interrupted. One hour after the last priming pulse of GnRH, 10 μg hu metastin 45–54 was administered as a bolus iv injection, and 1 h later the continuous iv infusion of hu metastin 45–54 (100 μg/h for 98 h) was initiated. The volume of infusion was monitored on a daily basis. During the last 3 h of the continuous hu metastin 45–54 infusion on d 4, the animals received, in sequence, a bolus injection of 10 μg hu metastin 45–54, a bolus injection of NMDA (10 mg/kg body weight), and a bolus injection of GnRH. In one monkey, the bolus injection of hu metastin 45–54 was administered after the GnRH and NMDA challenge. One day after termination of the continuous hu metastin 45–54 infusion, the animals received another iv bolus of 10 μg hu metastin 45–54. Finally, the intermittent priming infusion of GnRH was reinitiated.

Circulating concentrations of LH were monitored in blood samples collected on the following occasions. 1) On d 1, samples were collected before and after the last GnRH priming pulse and the bolus of hu metastin 45–54 (during these times, series of blood samples were collected 10 min before and at 10, 20, 30, and 50 min after the peptide bolus). Samples were also collected on d 1 during the first 12 h of the continuous hu metastin 45–54 infusion (at 10, 20, 30, 50, 70, 90, 110, 130, 150, 170, 360, and 720 min into the infusion). 2) On d 2, 3, and 4 of the continuous hu metastin 45–54 infusion, a single blood sample was collected in the morning and evening, at approximately 1000 and 2200 h, respectively. In addition, in two of three animals, a nocturnal series of blood samples was collected on d 2 at 20-min intervals over a 3-h period (1900–2200 h). 3) On d 4 of the continuous hu metastin 45–54 infusion, series of blood samples were collected to describe the LH response to bolus injections of hu metastin 45–54, NMDA, and GnRH. 4) One day after termination of the continuous hu metastin 45–54 infusion, a series of blood samples were collected before and after another iv bolus of hu metastin 45–54.

Nonheparinized blood samples were also collected in EDTA tubes before, during, and after the continuous hu metastin 45–54 infusion to measure peptide levels in the circulation at a later date. Plasma samples were stored at −20 C or below.

After a 1- to 3-wk interval, during which time the animals were reprimed with pulsatile GnRH, the control experiment was performed using a continuous infusion of vehicle (0.33% DMSO in sterile DPBS at 3 ml/h for 98 h) employing an essentially identical protocol.

LH assays

Plasma LH levels were measured using a homologous (macaque) RIA as described previously (21, 22). The sensitivity of the LH assay ranged from 0.36–0.42 ng/ml, and the intra- and interassay coefficients of variation for LH at 74% binding were less than or equal to 3.5 and 13.6%, respectively.

Statistical analysis

The significance of differences between mean LH concentrations were examined with the Student's *t* test, using Sigma Stat.

Results

Effect of continuous administration of hu metastin 45–54 on LH release in agonadal juvenile male monkeys

The last iv priming pulse of GnRH administered to three agonadal male monkeys at 0800 h on d 1 induced an LH discharge that increased circulating LH levels from 4.2 ± 0.7 ng/ml to a peak level of 5.8 ± 0.1 ng/ml (mean ± SEM; see Fig. 1). At 0900 h, the iv administration of 10 μg hu metastin 45–54 also elicited a rise in plasma LH levels, the amplitude (basal to peak, 4.7 ± 1.6 to 8.8 ± 1.7 ng/ml) of which was almost 2-fold that produced by the preceding bolus of GnRH. At 1000 h, continuous exposure to hu metastin 45–54 (100 μg/h) was initiated. Peak LH levels (10.6 ± 0.8 ng/ml) were observed at 1–2 h, and these then declined dramatically in the face of continuing exposure to the peptide, reaching, within 12 h, values (~1 ng/ml) indistinguishable from the control vehicle infusions. Interestingly, these very low LH levels were sustained during the day (1000 h), but in the evening (2200 h), modest, albeit nonsignificant, elevations were consistently observed during both continuous hu metastin 45–54 and vehicle infusions (Fig. 2).

Effect of single boluses of hu metastin 45–54, NMDA, and GnRH on LH release during the last 3 h of the continuous 4-d administration of hu metastin 45–54 in agonadal juvenile male monkeys

On d 4, single doses of hu metastin 45–54, NMDA, and GnRH were administered iv during the final 3 h of the hu metastin 45–54 infusion (Fig. 3). Although NMDA and GnRH elicited discharges of LH, hu metastin 45–54 did not. Twenty-one hours after termination of the metastin 45–54 infusion, however, a profound LH response was induced by administration of an identical bolus of the peptide.

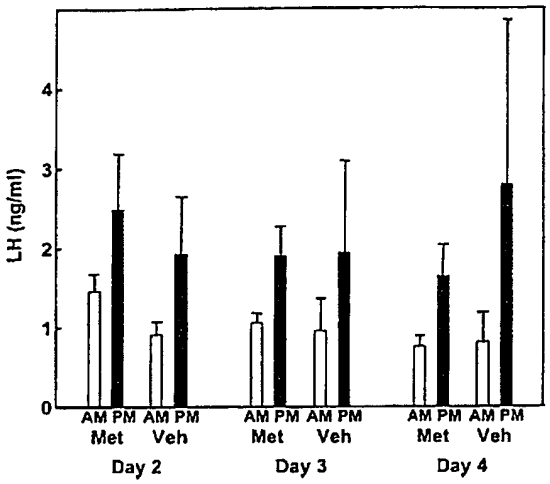

FIG. 2. Daytime (0900 h, *white bars*) and nighttime (2200 h, *black bars*) plasma LH levels (mean ± SEM) from three monkeys on d 2, 3, and 4 of a continuous infusion of 100 μg/h hu metastin 45–54 (Met) or vehicle (Veh). Daytime *vs.* nighttime differences were not significant by paired *t* test ($P > 0.05$).

Discussion

Reminiscent of intermittent GnRH infusions to hypothalamically lesioned monkeys (23), we recently demonstrated that intermittent pulsatile hu metastin 45–54 administration (2 μg per pulse at a circhoral frequency) drives gonadotropin secretion in agonadal, juvenile male monkeys previously primed with GnRH (17). In striking contrast, as shown in the present study with a similar experimental model, continuous

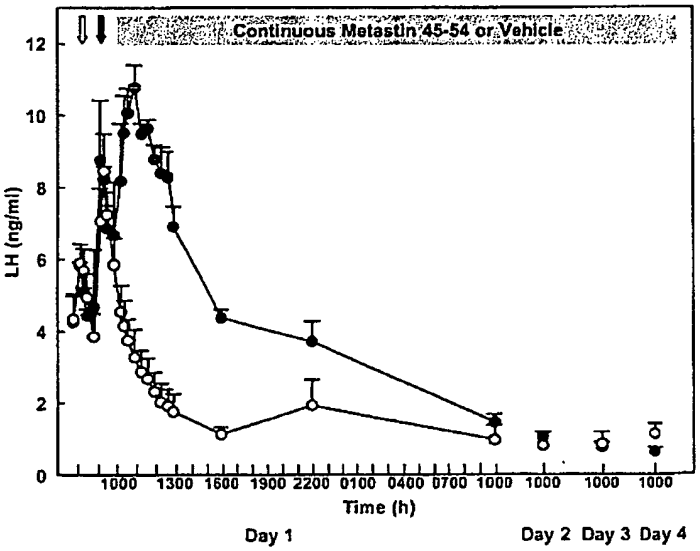

FIG. 1. Effect of continuous administration of hu metastin 45–54 on LH release in agonadal juvenile male rhesus monkeys, in which pituitary responsiveness to GnRH had been previously heightened by pulsatile GnRH treatment. ●, LH levels (mean ± SEM) from three monkeys receiving 100 μg/h hu metastin 45–54 over a 98-h infusion period (*shaded horizontal bar*); ○, mean LH levels during vehicle infusion. Administration of hu metastin 45–54 or vehicle was initiated at 1000 h on d 1. The *white arrow* indicates iv administration of the last GnRH priming pulse at 0800 h on d 1. The *black arrow* indicates iv administration of single bolus of hu metastin 45–54 at 0900 h on d 1.

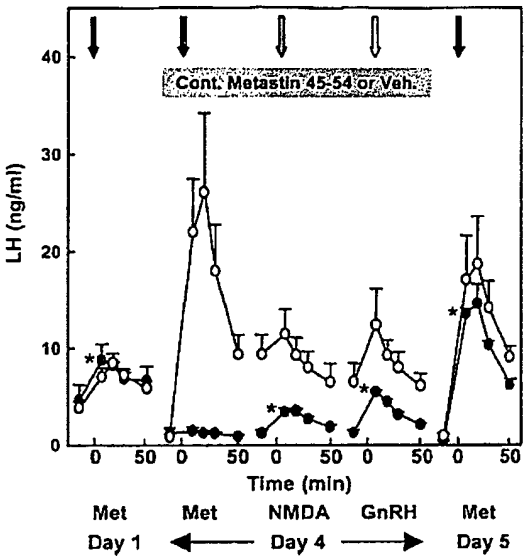

FIG. 3. Effect of single sequential boluses of hu metastin 45–54 (Met) (*black arrow*), NMDA (*gray arrow*), and GnRH (*white arrow*) on plasma LH concentrations (mean ± SEM) during the last 3 h of the 98-h iv infusion (*shaded horizontal box*) of hu metastin 45–54 at a dose of 100 μg/h (●) or vehicle (Veh) (○) compared with the LH response to the same bolus of hu metastin 45–54 1 h before (d 1) and 21 h after (d 5) the termination of continuous hu metastin 45–54 or vehicle infusion. *, Infusion of hu metastin 45–54 (●) significantly different ($P < 0.05$) from preinjection mean; n = 3.

administration of hu metastin 45–54 at 100 μg/h abolishes the LH response after an initial, acute stimulatory effect.

Possibilities to explain the observed decrease in LH levels in the face of continuous exposure to hu metastin 45–54 include desensitization of GPR54 to the exogenous metastin, depletion of GnRH from the GnRH neurons, desensitization of the GnRH receptor to GnRH, or depletion of LH from the pituitary gonadotrophs. Single boluses of several agonists were administered during the final day of the hu metastin 45–54 infusion to test these hypotheses. On d 4 of the hu metastin 45–54 infusion, administration of NMDA, an excitatory amino acid analog, evoked a robust LH response, demonstrating adequate stores of releasable GnRH within GnRH neurons, intact GnRH receptor signaling, and adequate stores of LH in the gonadotrophs. Administration of a 0.3-μg physiological pulse of GnRH also elicited a robust LH response (17), again testifying to the retained signaling capacity of the GnRH receptor and intact LH stores. However, administration of a single bolus of hu metastin 45–54 (concomitant with the continuous hu metastin 45–54 infusion), at a dose that elicited a robust LH discharge at the beginning of the experiment, failed to evoke a LH response. These data suggest that the inability to maintain LH levels 6 h after continuous hu metastin 45–54 infusion is due to desensitization or down-regulation of GPR54. Although the cellular mechanism for such an effect is currently unknown, sensitivity to exogenous hu metastin 45–54 was restored 21 h after its continuous administration was stopped.

Two other studies have used a metastin infusion paradigm. Fifty nanomoles of hu metastin 45–54 were infused intracerebroventricularly over 4 h to adult ovariectomized ewes treated with estradiol implants (15). Although GnRH levels in cerebrospinal fluid were sustained during the course of the infusion, LH levels appeared to wane by the end of the treatment period in some animals. If GnRH levels in cerebrospinal fluid accurately reflect those in portal blood, the latter finding suggests the development of gonadotroph refractoriness. This observation would then stand in contrast to our data indicating that pituitary responsivity to exogenous GnRH remained intact. In addition to species- and sex-specific mechanisms, differences between the two models include 1) the sex steroid milieu (the monkeys used in these experiments were juvenile and gonadectomized with no sex-steroid treatment) and 2) the dose and duration of metastin. In addition to the sheep study, recently, full-length hu metastin 1–54 was infused briefly (90 min) in healthy male volunteers (16). LH, FSH, and testosterone levels rose significantly, but additional interrogations of the hypothalamic-pituitary-gonadal axis were not performed.

Despite the dramatic drop in LH levels that occurred on d 1 of the hu metastin 45–54 infusion reported here, LH levels rose modestly on subsequent days during evening sampling compared with morning values, as they also did during vehicle infusion. Consistent nighttime elevations in LH have not previously been noted in agonadal, GnRH-primed juvenile males as young as 21–23 months of age (18), and therefore the present cohort of monkeys may have been more mature than those employed in earlier studies. In any event, nocturnal increases in LH as the first harbinger of the activation of the GnRH pulse generator have been observed in intact as well as gonadectomized male and female monkeys (24–26), normal pubertal children (27), and girls with gonadal dysgenesis (28). It is possible that the dose of hu metastin 45–54 administered (although seemingly high) was not adequate to achieve desensitization of GPR54 in all hypothalamic areas. Alternatively, it is possible that distinct circadian signals to GnRH-induced LH release exist that use pathways that do not include a metastin/GPR54 component. In this regard, it is to be noted that studies of the rat have shown that pretreatment with MK-801 (a NMDA receptor antagonist) fails to abrogate mouse metastin 43–52-induced LH release (29), and in the present experimental primate model, NMDA was able to elicit a LH response during the last day of the hu metastin 45–54 infusion. These findings suggest that glutamate and GPR54 ligands affect GnRH signaling through independent pathways.

Because GnRH can be used to stimulate the reproductive axis in hypogonadotropic states and GnRH receptor agonists and antagonists can be used to perform selective, reversible suppression of the axis in certain cancers, gynecological disorders, and precocious puberty, metastin's down-regulation of GPR54 opens new research opportunities for probing the hypothalamic-pituitary-gonadal axis as well as novel therapeutic possibilities for the treatment of reproductive disorders. Clearly, greater exploration of the physiological and pharmacological features of metastin will be required before this translation to clinical usefulness can occur, but the recent administration of hu metastin to men in a brief infusion (16) holds promise that this peptide can be administered without significant toxicities.

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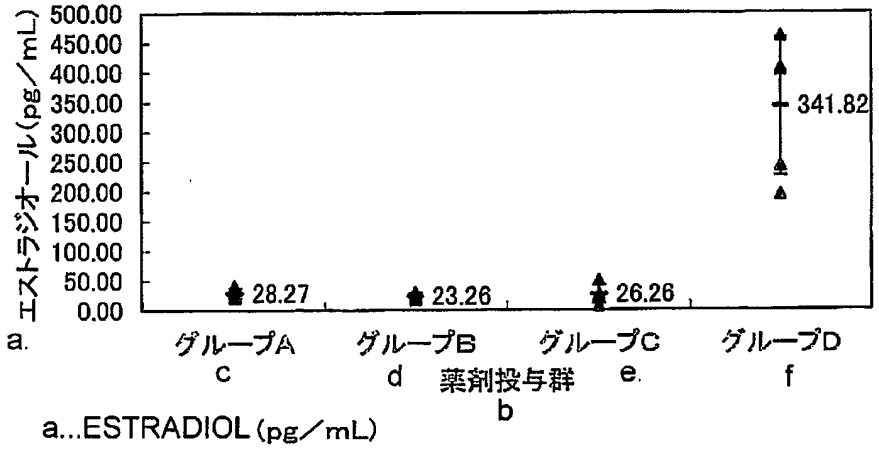
(54) 発明の名称: 性腺機能改善剤



a...ESTRADIOL (pg／mL)
b...DRUG ADMINISTRATION GROUP
c...GROUP A
d...GORUP B
e...GROUP C
f...GROUP D

(57) Abstract: Metastin and compounds promoting the activity of metastin or its receptor are useful as excellent gonadal function improving agents, ovulation inducers or promoters, gonadotropin secretion promoters, gonadotropin secretion inhibitors, sexual hormone secretion promoters, sexual hormone secretion inhibitors and so on to be used in preventives and remedies for infertility, hormone-sensitive cancer, endometriosis, etc. Metastin and its receptor are useful in screening these drugs.

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